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# **SOME ASPECTS OF THE PRODUCTION AND QUALITY OF FERMENTED MILKS USING BIFIDOBACTERIA**

by

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## ABSTRACT

Consumption of fermented milk products containing "probiotic" organisms is expanding in most countries around the world. The present study comprised two approaches: firstly, the production of set-type fermented milks using single strains of bifidobacteria and secondly the production of set-type fermented milks using commercial mixed starter cultures containing bifidobacteria. The former approach was unsuccessful because the products lacked the typical characteristics of a good fermented milk (*i.e.* they exhibited excessive whey leakage and weak gel formation). Consequently, attention was focused on evaluation of the quality of eight set-type fermented milks of which seven were made using commercial mixed starter cultures containing bifidobacteria and one using yoghurt starter culture.

The viable cell counts of micro-organisms in the eight starter cultures were evaluated by the plate count method. Morphological studies using confocal microscopy were performed on all of the micro-organisms in the starter cultures. Carbohydrate utilisation and enzymatic tests, using miniature reaction strips were also performed on all of the micro-organisms.

The products were made from reconstituted skimmed milk powder (SMP), milk protein concentrate (MPC), and anhydrous milk fat (AMF), and were inoculated with the eight different starter cultures, dispensed into cups and incubated at 40 °C until the pH was ~4.60. The fermented milks were then transferred to a cold store and evaluated when fresh and after storage for 20 days.

The recovery of viable counts of micro-organisms in the eight starter cultures agreed with the suppliers' specifications. The morphological studies using confocal microscopy allowed identification of the micro-organisms at generic level. Thus, it was confirmed that the single strain isolates were typical of bifidobacteria, lactobacilli and streptococci.

The enzymatic activity tests generally, confirmed the identity of the isolates of bifidobacteria in the starter preparation. The isolates from starter cultures MSK 2, AC/BL and DV B-100 were identified as *Bif. longum*, *Bif. infantis* and *Bif. bifidum* respectively. Isolates of *Bifidobacterium* from starter cultures AB, ABT-1, ABT-3 and MSK B2 were not identified. However, isolates of *Lb. delbrueckii* ssp. *bulgaricus* from starter culture MY 087 were identified as *Lb. delbrueckii* ssp. *lactis* which was subsequently confirmed by the manufacturer. The tests confirmed the identity of all the *Lb. acidophilus* and *Str. thermophilus* isolates.

The gross chemical composition of the fermented milks produced using the mixed starter cultures was similar, but the organic acids profile was different. The fermented milks made using AB and AC/BL starter cultures (*i.e.* containing bifidobacteria and *Lb. acidophilus*) had a higher content of acetic acid compared to the rest which contained the same organisms in addition to *Str. thermophilus*.

The growth of bifidobacteria was inhibited by the presence of *Str. thermophilus* in starter cultures. However with the exception of MSK 2 and ABT-1 the number of viable cells remained at acceptable levels (*i.e.*  $>10^6$  cfu g<sup>-1</sup>) after the storage period. A large number of viable cells of bifidobacteria and *Lb. acidophilus* in the products AB and AC/BL was observed, suggesting a possible associative growth between them.

The viable cell counts of *Lb. acidophilus* were within acceptable levels (*i.e.*  $>10^6$  cfu g<sup>-1</sup>) after the storage period, but the product made with starter culture MSK B2 was the exception. The count of *Lb. delbrueckii* ssp. *bulgaricus* from starter culture MY 087 fell to the borderline ( $\sim 10^6$  cfu g<sup>-1</sup>) after the storage period. However, the viable cell counts of *Str. thermophilus* were very high for all the products after the storage period.

The rheological properties (firmness and syneresis) followed similar patterns for all the fermented milks. Syneresis decreased while the firmness increased with time.

Sensory evaluation clearly differentiated the three products made with starter cultures AB, AC/BL and MSK 2 from the rest of the fermented milks. The first two were noted for their acid and vinegar character and MSK 2 for being the least acid of the products.

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## **CHAPTER ONE:**

## **INTRODUCTION**

## CHAPTER ONE: INTRODUCTION

When and where the production of fermented milk products was first initiated is unknown. However, there is a little doubt that the consumption of yoghurt and other fermented milks dates back to the domestication of animals (cow, goat, sheep or buffalo) by man and the beginning of agriculture (Helferich and Westhoff, 1980). According to some sources it is believed that the first sour milk originated in Asia, where the ancient Turks lived as nomads. In the 8th century, the Turks called to such product 'Yogurut' which was subsequently changed in the 11th century to its present name, 'Yoghurt'. Others have suggested that yoghurt originated in the Balkans, and the inhabitants of Thrace, who were known for breeding large flocks of sheep. They used to make sour milks called 'Prokish' which later became yoghurt (Rasic and Kurmann, 1978).

The classical story about the 'birth' of yoghurt was reported by Helferich and Westhoff (1980). The milk from animals (camel, buffalo, goat, sheep or cow) was stored in an animal skin bag which was placed close to its body. The heat from the animal's body maintained the optimum conditions for growth of micro-organisms present in milk, mainly lactic acid bacteria. Several hours later when the nomad prepared to drink milk, he was surprised to find a semisolid mass coagulum. When he consumed this new substance, he found the taste to be delightful. The most important advantage of such new product was that it could be stored in warm climates and be safely consumed for several days.

Originally, fermented milks were obtained by mixing a small quantity of sour milk, which was soured naturally, with fresh warm milk. The next stage of development to produce fermented milk was using boiled milk that was partly concentrated in order to get a thicker coagulum. Then the milk was cooled, seeded with fermented milk from the previous day, and left for few hours to acidify and form a gel (Robinson and Tamime, 1990).

Ancient physicians of the Near and Middle East prescribed yoghurt or soured milks for curing disorders of the stomach, intestines and for stimulating the appetite (Rasic and Kurmann, 1978). However, at the beginning of the 20th century, Metchnikoff (1910), who more than anyone influenced the study of fermented milk, observed and later reported that Bulgarians had an average of 87 years of live expectancy (Helferich and Westhoff, 1980). The longevity of these people was due to the benefits of consumption of fermented milk products, made with an organism identified as *Bulgarian bacillus* which was later designated as *Lactobacillus delbrueckii ssp. bulgaricus*.

Metchnikoff's theory, which advocated that milk fermented with this *Lactobacillus*, had a favourable influence upon the intestinal flora, was challenged in 1915 by Rabe who demonstrated that these micro-organisms do not survive passage through the stomach and small intestine (Ballonge, 1993). A decade later Retger and Cheplin (1921) found out that the bacteria that can establish itself in the intestine was *Lactobacillus acidophilus*, and the attributed therapeutic value of yoghurt was induced when *Lb. acidophilus* was one of the bacteria in the starter culture (*i.e.* *Lb. delbrueckii ssp. bulgaricus* and *Streptococcus thermophilus*).

Thus, the possible prophylactic and/or therapeutic properties of yoghurt and related products have been the subject of controversy and speculation (Tamime *et al.*, 1995). All of these controversial theories stimulated a large amount of research over the years, and without doubt, have influenced the popularity and spread of yoghurt and related products in most countries.

Consequently, milk technologists, nutritionists and clinicians, turned their attention to other micro-organisms, known as 'probiotics' which can colonise the intestinal tract. Some examples include *Lb. acidophilus*, *Bifidobacterium* species and other lactic acid bacteria. According to Gurr *et al.* (1984) "the micro-organisms with the best chance of passing through the stomach and small intestine, and colonising the medium are those endogenous to the species consuming the fermented product". Therefore, research has been focused on the genus *Bifidobacterium*, which unlike the

bacteria of yoghurt, are isolated from animals and humans. At present, twenty nine strains of bifidobacteria have been isolated and only five have attracted interest in the dairy industry. These isolates are *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium adolescentis*, *Bifidobacterium longum* and *Bifidobacterium infantis*.

There is a wide range of lactic acid bacteria namely: *Lb. delbrueckii* ssp *bulgaricus*, *Lb. acidophilus*, *Str. thermophilus*, *Lactobacillus rhamnosus*, *Lactobacillus paracasei* ssp. *paracasei* and others, which are considered to possess beneficial effects in the human intestinal tract. Although, the probiotic and therapeutic properties have been attributed to bifidobacteria, this seems to suggest imminently the beneficial health effects. However, the pharmaceutical and food industries, more specifically the milk industry, need to fill the gaps in relation to the quality of these products containing novel micro-organisms which are offered to the consumer.

No data is available for the production and quality of commercial fermented milk using single strains of bifidobacteria, and for this reason the aspects evaluated in this thesis could be summarised as follows:

- a) the use of 5 different single strains of bifidobacteria of human origin for the production of set-type fermented milk, and the effect of these micro-organisms on the quality of such product (*i.e.* rheological and sensory properties);
- b) the rate of acidification of milk when using these strains of starter cultures, and their survival rate after storage for 21 days;
- c) the determination or identification of some of the flavour characteristics of these fermented milks; and
- d) the correlation of rheological measurement of fermented milk with the sensory attributes.

Preliminary studies were not successful. The rate of acidification of the milk was variable when using the same single strain of starter cultures, and in some occasions the gel had a 'cheesy' smell. The processing conditions, for example: (a) addition of growth factor to the milk base, (b) heat treatment of the milk to a higher temperature and (c) fortification of the milk base with high protein skimmed milk

powder, were modified but the end product still lacked the characteristics of a typical fermented milk gel. Thus, as a consequence the single strains were replaced by 7 mixed commercial bio-starter cultures, and using a yoghurt culture as a control. However, the above parameters have been maintained, and other aspects studied included:

- (a) the enumeration of the different microflora present in the fermented milks, and to monitor their survival after storage;
- (b) the determination of the degree of denaturation of the proteins after the heat treatment of the milk base;
- (c) the identification and partial characterisation of the individual micro-organisms in the commercial starters used.

## **CHAPTER TWO:**

## **LITERATURE REVIEW**



## CHAPTER TWO: LITERATURE REVIEW

### 2.1 Historical Background of Bifidobacteria

Bifidobacteria were first isolated at the beginning of this century by Tissier (1900) from the faeces of breast-fed infants. The bacterium was a rod shaped, and was named *Bacillus bifidus*. The genus *Bifidobacterium* was first described by Orla-Jensen (1924) who had given a decisive shift in the history of bacterial taxonomy. He proposed the transfer of the name *Bacillus bifidus* to *Bifidobacterium bifidum*. However, in spite of this proposal, the name of *Lactobacillus bifidus* was reported in the 7th edition of Bergey's Manual of Determinative Bacteriology (Breed *et al.*, 1957).

The first differentiation characteristics of bifidobacteria were contributed by Dehnert (1957) who classified them into five groups. Followed by the description of species according to their source of natural habitat (*i.e.* from human intestinal tract, from alimentary tracts of animals and bees) ( Reuter, 1963; Mitsuoka, 1969; Scardovi *et al.*, 1969; Scardovi and Trovatelli, 1969).

In 1974, the 8th edition of Bergey's Manual of Determinative Bacteriology recognised *Bifidobacterium* as a genus consisting of eleven species (Buchanan and Gibbons, 1974). However, in the latest edition of Bergey's Manual, Scardovi (1986) reported 24 species in the genera *Bifidobacterium* which are grouped according to their origin (*i.e.* human and not human). Fifteen isolates originated from animals and nine from natural cavities of human being. Recently, an additional five species have been described (Sgorbati *et al.*, 1995) making up a total of 29 species; ten of which are of human origin (intestine, vagina and oral cavity) and the remainder from animals, insects and sewage.

The therapeutic effect of fermented milk known as yoghurt in the Balkans was attributed to *Lb. delbrueckii* ssp. *bulgaricus* (Metchnikoff, 1908), and latter to *Lb. acidophilus* by Rabe (1915) and Retger and Cheplin (1921). Since bifidobacteria, *Lb. acidophilus* and other lactic acid bacteria are among the micro-organisms present in

the human intestine and possess beneficial effects, many researches have been investigating the health promoting factors of such probiotic micro-organisms. Some of the benefits include the improvement of lactose digestion and the anti-diarrhoea effect (Mogensen, 1995). Nevertheless, other health benefits such as, anti-cholesterol effects, stimulation of the immune system and anti-carcinogenic effects are also attributed to these organisms. Fuller (1989) defined a probiotic culture as follows: "a live microbial feed supplement which beneficially affects the host of animal by improving its intestinal balance".

Given the possible therapeutic action of bifidobacteria species, the pharmaceutical and dairy industries started using these cultures to manufacture medical products and fermented milks, as possible vehicle to colonise the human gastrointestinal (Rasic and Kurmann, 1983). For example, in Germany milk products containing bifidobacteria were introduced in the 1960s (Peitersen, 1991).

## **2.2 Definition and Classification of Fermented Milks**

According to the International Dairy Federation (IDF, 1992a) the definition of fermented milks is as follows: "Fermented milks are prepared from milk and/or milk products by the action of specific micro-organisms which result in reduction of pH and coagulation. These micro-organisms must be viable, active and abundant in the finished product at the time of sale for consumption".

The micro-organisms, which are widely employed in the manufacture of fermented milks, are either bacteria, yeasts and moulds or combinations of these. Table 2.1 lists these micro-organisms referred to as dairy starter cultures. In general, the major metabolites produced by the micro-organisms during the fermentation of milk could be summarised as follows:

- L(+), D(-) or DL lactate
- Diacetyl, acetaldehyde, acetoin
- Acetate
- Ethanol, carbon dioxide

**Table 2.1** Starter cultures for milk fermentations and their principal metabolic products.

Starter organism	Important metabolic products
<b>I. Lactic acid bacteria</b>	
<b>A. Mesophilic</b>	
<i>Lactococcus lactis</i> ssp. <i>lactis</i>	L(+) lactate
biovar. <i>diacetylactis</i>	L(+) lactate, diacetyl
ssp. <i>cremoris</i>	L(+) lactate
<i>Leuconostoc mesenteroides</i> ssp. <i>mesenteroides</i>	D(-) lactate, diacetyl
ssp. <i>cremoris</i>	D(-) lactate, diacetyl
ssp. <i>dextranicum</i>	D(-) lactate, diacetyl
<i>Pediococcus acidilactici</i>	DL lactate
<b>B. Thermophilic</b>	
<i>Streptococcus thermophilus</i>	L(+) lactate, acetaldehyde, diacetyl
<i>Lactobacillus delbrueckii</i> ssp. <i>delbrueckii</i>	D(-) lactate
ssp. <i>bulgaricus</i>	D(-) lactate, acetaldehyde
ssp. <i>lactis</i>	D(-) lactate
<i>Lactobacillus fermentum</i>	DL lactate
<i>Lactobacillus helveticus</i>	DL lactate
<i>Lactobacillus kefir</i>	DL lactate
<i>Lactobacillus kefiranoferens</i>	DL lactate
<b>C. Therapeutic</b>	
<i>Lactobacillus acidophilus</i>	DL lactate
<i>Lactobacillus paracasei</i> ssp. <i>paracasei</i>	L(+) lactate
ssp. <i>shirota</i>	L(+) lactate
<i>Lactobacillus rhamnosus</i>	L(+) lactate
<i>Lactobacillus reuteri</i>	DL lactate, CO <sub>2</sub>
<i>Bifidobacterium adolescentis</i>	L(+) lactate, acetate
<i>Bifidobacterium bifidum</i>	lactate, acetate
<i>Bifidobacterium breve</i>	L(+) lactate, acetate
<i>Bifidobacterium infantis</i>	lactate, acetate
<i>Bifidobacterium longum</i>	L(+) lactate, acetate
<b>II. Miscellaneous bacteria</b>	
<i>Acetobacter aceti</i>	
<b>III. Yeasts</b>	
<i>Candida kefir</i>	Ethanol, CO <sub>2</sub>
<i>Saccharomyces unisporus</i>	
<i>Saccharomyces cerevisiae</i>	Ethanol, CO <sub>2</sub>
<i>Saccharomyces exiguus</i>	
<i>Kluyveromyces marxianus</i>	
<b>IV. Moulds</b>	
<i>Geotrichum candidum</i>	

After Tamime and Marshall (1997).

- Miscellaneous (*e.g.* exopolymer, organic acids, fatty acids)

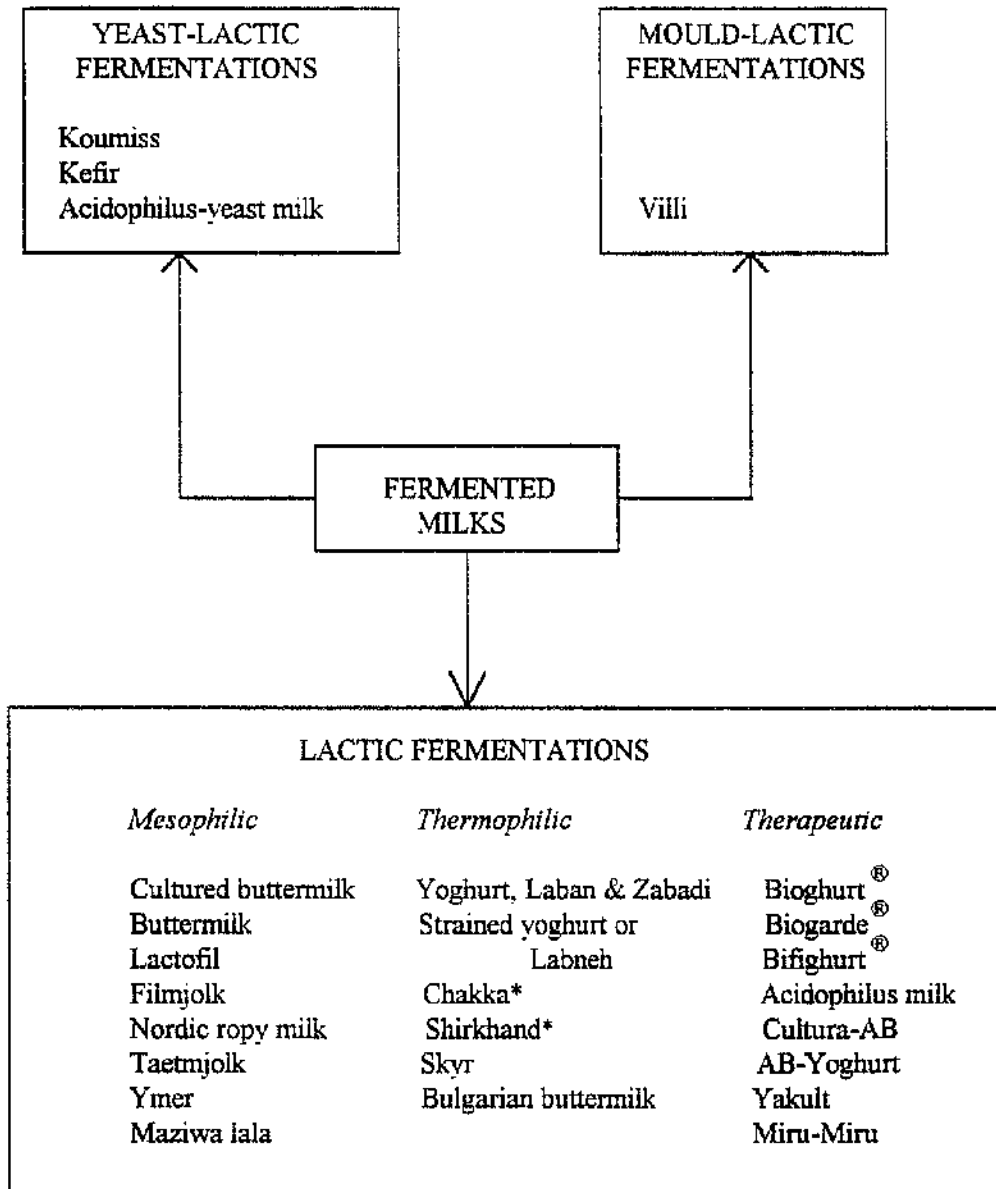
Around 400 generic names have been applied to traditional and industrialised fermented products in different parts of the world (Kurman *et al.*, 1992; Tamime *et al.*, 1995). Whilst these products may have different local names, they may be very closely related or practically the same. Thus, an approach for the classification of fermented milks should take into account the type of milk used and more appropriately the micro-organisms which dominate the flora. According to Robinson and Tamime (1990) these products have been divided in three broad categories based on the metabolites produced which are: (a) lactic acid fermentation, (b) lactic acid fermentation with yeast and (c) lactic acid fermentation with mould. A generalised scheme of classification of fermented milks is shown in Figure 2.1 including some typical examples. Some of these products are manufactured from fermented milk by de-wheyling to concentrate the product (*e.g.* Labneh, Ymer, Skyr or Shirkhand).

### 2.3 Patterns of Production and Consumption

There are no exact data available in the dairy industry to indicate the production and consumption figures of different fermented milk products in the world. However, the statistical figures published by the International Dairy Federation include yoghurt and other fermented milks. Table 2.2. shows the pattern of fermented milks consumption in some selected countries since 1980. It is evident that the popularity of fermented milks in many countries may be, in part, attributed to tradition, but their overall properties (*e.g.* flavour, aroma, texture and health aspects) are more attractive to consumers rather than fresh milk.

The consumption figures of fermented milks in the Balkans, Turkey and the Middle Eastern countries are not available although such products are widely consumed. However, yoghurt and yoghurt-related products are widely consumed throughout the world (Table 2.2) and, for example, the per capita consumption figures in the Scandinavian countries is reported as the highest in the world (*e.g.* Finland 37 kg per head per annum in 1993).

**Figure 2.1** Scheme for classification of fermented milk products.



\* In some instances mesophilic cultures are employed.

After Robinson and Tamime (1990).

**Table 2.2** Statistical data of consumption of yoghurt and fermented milk products.

Country	1980		1985		1990		1993	
	Yoghurt	Others	Yoghurt	Others	Yoghurt	Others	Yoghurt	Others
Australia	1.9	-	2.8	-	3.5	-	4.8	-
Austria	5.8	2.0	6.6	2.2	7.5	-	8.6	2.5
Belgium	4.9	-	6.0	0.06	6.5	1.2	5.7	3.9
Canada	1.7	-	2.5	-	3.2 <sup>a</sup>	-	3.05	-
Chile	1.4	-	2.5	-	3.9	-	4.10 <sup>b</sup>	-
Czechoslovakia	1.7	2.5	2.5	3.1	3.3 <sup>c</sup>	3.3 <sup>c</sup>	3.4 <sup>d</sup>	5.1 <sup>d</sup>
Denmark	9.7	6.3	8.0	7.5	7.8	6.9	8.3	6.8
Finland	8.4	28.5	9.4	28.0	11.4	25.4	13.3	23.7
France	9.4 <sup>e</sup>		12.7 <sup>e</sup>		16.4 <sup>e</sup>		17.3 <sup>e</sup>	
Germany	6.7	1.2	7.9	1.1	10.6	0.8	11.3	0.9
Hungary	0.9	1.0	1.1	1.3	1.0	1.4	2.1 <sup>f</sup>	1.5
Iceland	5.9	14.4	6.4	14.5	9.9	14.7	9.8	16.1
Ireland	2.3	-	3.5	-	3.1	-	3.7 <sup>d</sup>	-
Israel	4.7	9.6	8.1	8.7	-	-	10.7 <sup>d</sup>	10.4 <sup>d</sup>
Italy	1.3	0.2	1.6	1.3	2.6	1.4	5.0 <sup>e</sup>	
Japan	2.2	4.3	2.9	4.4	3.9	3.9	4.8	3.7
Luxembourg	5.1 <sup>e</sup>		6.8 <sup>c</sup>		6.1 <sup>e</sup>		-	
Poland	0.1	0.7	0.4	0.6	-	-	-	-
Netherlands	16.9	-	18.1	-	21.8	-	20.7	-
Norway	2.2	12.9	3.1	10.9	4.3	10.6	6.3	-
South Africa	-	-	1.4	2.7	1.6	2.0	1.8	1.75
Spain	6.0	-	5.5	-	8.0	-	9.8 <sup>e</sup>	
Sweden	4.2	19.7	4.7	21.9	7.4	21.7	7.5	21.1
Switzerland	13.8	-	16.2	-	17.3	-	17.0	-
USA	1.2	-	1.8	-	-	-	2.1	-
United Kingdom	2.1	-	3.1	-	4.3	0.1	4.6	0.2

<sup>a</sup> Data correspond to 1989.

<sup>b</sup> Consumption data for 1991.

<sup>c</sup> Consumption data for 1989.

<sup>d</sup> Consumption data for 1992.

<sup>e</sup> Data correspond to both products.

<sup>f</sup> Estimated.

- No data available.

Source: IDF (1982a, 1987a, 1992b, 1995).

Since yoghurt has been accepted and has become a very popular fermented milk product in the majority of countries, it has been used as a successful vehicle for the implantation of *Lb. acidophilus* and *Bifidobacterium* species in the gastrointestinal tract. The reason for such approach, is as follows : (a) these organisms have slow growth in milk and longer production time is required which may not be favourable, (b) acetic acid and other metabolites produced by these cultures gives the fermented milk different characteristics which may not be accepted by the consumers, and (c) milder acidic taste when compared with yoghurt.

Over the past decade fermented milk products containing *Lb. acidophilus* and *Bifidobacteria* (*Bif. bifidum*, *Bif. longum* or *Bif. breve*) have been used in mixed lactic starter cultures (mesophilic or thermophilic) including *Pediococcus acidilactici* in most European countries. Some illustrated examples are shown in Table 2.3.

## 2.4 Technology of Manufacture

Many researchers have extensively studied the technological, biochemical and physico-chemical aspects that occur during the manufacture of fermented milk products, including the compositional variations (e.g. lactation, season of the year, health of the cow, nutrition, duration between milking) which can affect the quality of the finished product (Rasic and Kurmann, 1978; Tamime and Robinson, 1985; Robinson and Tamime, 1993). However, Tamime *et al.* (1995) stated that the main stages of production of any type of fermented milk (i.e. standardisation of the fat content, fortification of the milk solids, homogenisation, de-aeration (optional) and heat treatment) have much in common. Thus, technical aspects which must be considered in detail, include : (a) the organisms that constitute the starter culture, (b) temperature and period of incubation, (c) the inoculation rate, and (d) production of bulk starter (some times not required). The industrial procedure for the manufacture of set and stirred fermented milk products including yoghurt is shown in Figure 2.2.

Table 2.3 Commercial and developed fermented milk products containing bifidobacteria.

Product	Microflora <sup>a</sup>											
	1	2	3	4	5	6	7	8	9	10	11	12
Cultura-AB <sup>®</sup> , Biomid <sup>®</sup> , Diphilus <sup>®</sup> , and Lünebest <sup>®</sup>	✓							✓				
Acidophilus bifidus yoghurt	✓ ↔ <sup>d</sup>	✓				✓	✓	✓				
Bifidus Active (BA <sup>®</sup> )		✓				✓	✓					
Bifidus milk	✓ ↔	✓										
Bifidus milk with yoghurt flavour	✓ ↔	✓ ↔	✓									
Bifidus yoghurt <sup>b</sup> , Mil-Mil B <sup>®</sup> or Biobest <sup>®c</sup>	✓ ↔	✓				✓	✓					
Bifighurt <sup>®</sup>		✓				✓						
Biogarde <sup>®</sup> or ABT	✓					✓		✓				
BioKys <sup>®</sup>		✓						✓			✓	
Biomild <sup>®</sup>					✓			✓				
Mil-Mil <sup>®</sup>	✓			✓				✓				
Offhus <sup>®</sup> 'nature'	✓					✓		✓				
'double douceur'								✓		✓		
Progut <sup>®</sup>		✓						✓	✓	✓	✓	
BRA			✓					✓				✓

<sup>a</sup> 1 → 4, *Bif. bifidum*, *longum*, *infantis*, *breve*, respectively; 5, *Bifidobacterium* spp. (not identified); 6, *Str. thermophilus*; 7, *Lb. delbrueckii* ssp. *bulgaricus*;

8, *Lb. acidophilus*; 9, *Lac. lactis* biovar *diacetylactis*; 10, *Lac. lactis* ssp. *cremoris*; 11, *P. acidilactici*; 12, *Lactobacillus reuteri*.

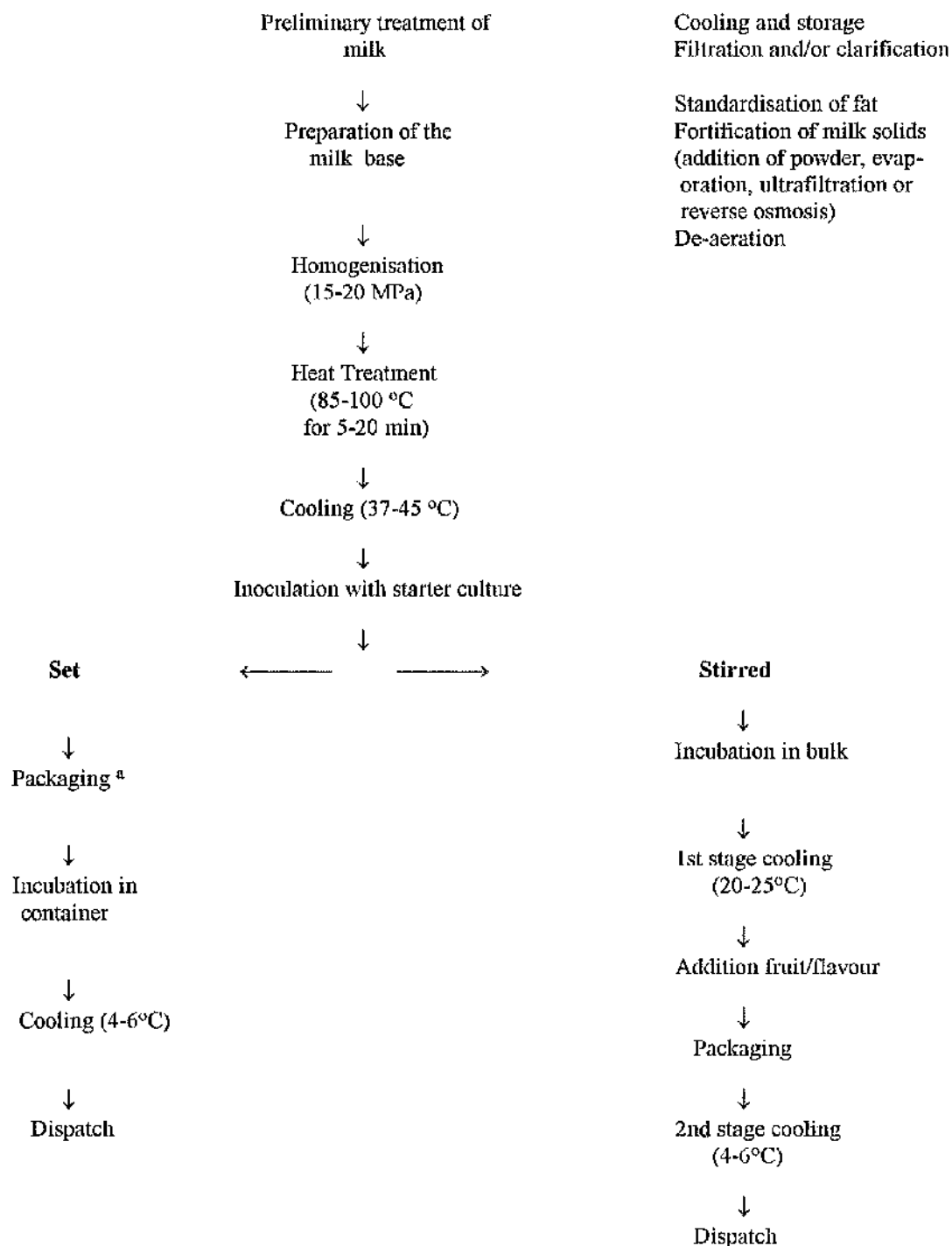
<sup>b</sup> With or without *Lb. acidophilus*.

<sup>c</sup> The product contains 'biogerm' grains and fruits.

<sup>d</sup> Indicates that either species have been used.

After Tamime and Marshall (1997).



**Figure 2.2** The major stages for production of fermented milk products.<sup>a</sup> Sucrose and/or fruit can be added at this stage.

### 2.4.1 Raw material

The chemical composition and the microbiological quality of the raw milk can affect the quality of fermented dairy products. According to Rasic and Kurmann (1978) the milk intended for the manufacture of fermented milk products should be:

- Free from inhibitory compounds, which can inhibit the growth of starter culture (*e.g.* antibiotics).
- High level of total solids, specially proteins which play an important role on the consistency of the product and prevents whey separation; the presence of fat in milk enhances the taste of the product.
- Milk from healthy cows, because milk from abnormal udder (*i.e.* mastitis) can influence the quality of the finished product.

In general, the chemical composition of milk is affected by many factors such as type of mammals, breed, stage of lactation, climatic conditions, diet, and season of the year (Juarez and Ramos, 1984; Tamime and Robinson, 1985). Table 2.4 shows the chemical composition of milk from different species of mammals (Tamime and Robinson, 1985).

**Table 2.4** Chemical composition ( $\text{g } 100\text{g}^{-1}$ ) of milk of different species of mammals.

Species	Fat	Protein	Lactose	Ash	Water
Buffalo	8.0	4.2	4.9	0.8	82.1
Cow	3.8	3.3	4.7	0.7	87.6
Camel	4.2	3.7	4.1	0.9	87.1
Goat	4.5	3.3	4.6	0.6	87.0
Sheep	7.5	5.6	4.4	0.9	81.6

Adapted from Tamime and Robinson (1985).

### 2.4.2 Preliminary treatment of milk

It is well known that raw milk received from farms may contain foreign particles (hair, straw, and/or dust), impurities and cellular material such as leukocytes and epithelial cells. In addition, in developing countries, specially those in warm climatic conditions, milk reception in factories may be at temperatures as high as 20°C, as against a recommended temperature of <10°C. However, the industrial handling of milk is as follows: (a) metering or weighing, (b) filtering or centrifugal clarification, and (c) cooling to ~4°C using a plate cooler before storing in a silo (Tamime and Kirkegaard, 1991).

### 2.4.3 Preparation of the milk base

The chemical composition of the final product should comply national standards. The specifications may include a minimum or range of fat and solids-non-fat (SNF) contents. Thus, it is necessary to standardise the fat and the SNF contents of the milk base which can be achieved as follows:

*First:* The fat content in milk can be standardised using several methods such as: (a) removal of part of the fat content from milk, (b) mixing whole milk with skimmed milk, and (c) addition of cream or anhydrous milk fat to skimmed milk (fresh or recombined). For example, in the United Kingdom the average fat content in milk ranges between 3.8 to 4.2g 100g<sup>-1</sup>, and the minimum SNF ~8.5g 100g<sup>-1</sup>, but the fat content of the commercial yoghurts is between 0.5 to 1.5g 100g<sup>-1</sup> for low or medium fat type, and 10g 100g<sup>-1</sup> in Greek style yoghurt (Tamime *et al.*, 1987; Robinson and Tamime, 1990).

*Second:* The consistency of fermented milk products is influenced by the protein level in the milk, and also minimises syneresis (whey separation) after the fermentation stage. This can be achieved by: (a) the addition of powders (full cream milk, skimmed milk, whey, caseinates and/or high protein powders or retentate powders), (b) concentration of the milk base by evaporation, ultrafiltration or reverse osmosis, and (c) a proper mechanical handling of the coagulum after the fermentation stage.

Tamime *et al.* (1984) suggested that the casein level in the milk base is critical in relation to the strength of the coagulum, and recommended a ratio of casein to non-casein nitrogen of  $\sim 3.4$ . Such milk provides the necessary growth factors of *Lactobacillus* spp. and *Str. thermophilus*, but when using bifidobacteria, an increase in the milk protein hydrolysates seems to speed up their growth (Hunger and Peitersen, 1992). Other growth promoter factors such as yeast extract, vitamins, oligosacharides have also been studied [see the review by Tamime *et al.* (1995) for further details].

The incorporation of air to the milk base during the different processing stages should be avoided. Taking into consideration the variable sensitivity of bifidobacteria to oxygen, ideally a de-aeration process should be carried out.

#### 2.4.4 Homogenisation

The process of homogenisation is characterised by breaking up the milk fat globules to a smaller size, and as a consequence, the fat in the milk base does not rise to the surface. During the homogenisation process, the milk flows through series of restricting valves under high pressure which cause reduction of fat globule size from  $\sim 2\text{-}10\text{ }\mu\text{m}$  to  $\sim 0.7\text{-}1\text{ }\mu\text{m}$  (Helferich and Westhoff, 1980; Kessler, 1981).

Homogenisation is usually carried out prior to the heat treatment of the milk base, and the optimal pressure and temperature used may range between 15-20 MPa and 50-60 °C, respectively (Rasic and Kurman, 1978). According to Tamime and Robinson (1985, 1988b) and Robinson and Tamime (1993) the effect of homogenisation of the milk base demonstrates the following advantages:

- Prevents the fat globules from coalescing and rising to the surface.
- Minimises whey separation (syneresis) due to the protein-protein interaction, and as a result the water holding capacity is improved.
- Increases the viscosity of the product, as a consequence of the small fat globules are adsorbed onto the casein micellar structure of the coagulum.
- Breaks up the non-dissolved particles that eventually are added as ingredients.

- Improves light scattering, and the final product becomes whiter because of the increase in the number of small fat globules.
- Increases the digestibility of the product.

#### 2.4.5 Heat treatment

The temperature-time relationship of heat treatment of the milk base can vary from ordinary pasteurisation (72 °C for 15 s) to as high as 133 °C for 1 s (UHT) (Robinson and Tamime, 1990). However, the same authors stated that, for industrial purposes it ranges between 85-95 °C and for 5 to 30 min.

Heat treatment induces a multitude of changes to the milk base which have been reported by many researchers (Rasic and Kurmann, 1978; Tamime and Deeth, 1980; Puhani, 1988; Dannenber and Kessler, 1988a, 1988b; Tamime and Robinson, 1988a, Robinson and Tamime, 1993). A summary of these changes is:

- Destruction of pathogenic organisms and reduction of number of other undesirable micro-organisms.
- Improvement of the keeping quality and the quality of the product in general.
- Formation of growth promoting substances to certain starter cultures, *e.g.* formic acid.
- Inactivation of certain enzymes in milk such as lipase.
- Reduction of the amount of oxygen, so providing micro-aerophilic conditions required by the starter micro-organisms.
- Improvement of the consistency and viscosity of yoghurt, and prevention of whey separation caused by denaturation of the whey protein and their interaction with  $\kappa$ -casein
- Production of volatile compounds which can contribute to the flavour of the final product.
- Re-distribution of minerals, specially calcium, between the soluble and colloidal forms which may lead to reduce the coagulation time.

#### 2.4.6 Starter culture technology

Tamime *et al.* (1995) reported that the traditional scale-up system (*i.e.* stock culture → mother → intermediate → bulk) for the propagation of starter cultures for the manufacture of fermented milk products is being replaced by dried-to-vat inoculation (DVI) of bulk starter or the process milk. Bulk starter milk could be prepared by reconstituting antibiotic-free skimmed milk powder (total solids 10-12%), and heating to 90-95 °C for 15-30 minutes. Nevertheless, bifidobacteria grow to a limited extent in milk, and it is necessary to promote their growth by the addition of growth-enhancing substances such as glycoproteins and oligosaccharides. This method of bulk starter production may involve a risk of contamination due to the slow rate of such micro-organism and the appreciable high cell count may not be achieved (Hunger and Peitersen, 1992). Thus, at present the trend is to use DVI starters (deep-frozen or freeze-dried) either for the bulk starter production or for the manufacture of fermented milks. In the former approach care should be exercised not to upset the balance of cultures in a mixed culture system.

#### 2.4.7 Fermentation process

There are several different types of fermentation reaction that can occur in milk as a result of the biological activity of the micro-organisms and their enzymes. The biological activity that may occur in milk depends on the type of micro-organism used for the fermentation. The role of the lactic acid bacteria in the fermentation process in terms of their biological and physical activity, could be summarised briefly as follows:

- Lactose is utilised for respiration and for cell division, and such metabolic activity yields the production of lactic, acetic and other organic acids.
- During the fermentation process the growth of contaminating micro-flora is inhibited due to the production of antibacterial substances.
- Production of flavouring compounds (*e.g.* acetaldehyde, acetone, acetoin, and others) are essential for the aroma and flavour of the product.
- Some strains of micro-organisms produce polysaccharide material which helps to increase the viscosity of the gel and prevents syneresis.

- The acid formation helps to transform liquid milk into a gel, possibly because of several physico-chemical changes of the casein micelle which may involve demineralisation, aggregation, contraction and charge neutralisation at the isoelectric point (Heertje *et al.*, 1985).

The fermentation process of the stirred-type fermented milks occurs in bulk tanks, so the coagulum is broken before or during the cooling and the packaging stages. In contrast, the fermentation process of set-type fermented milks is achieved in the retail container, and the coagulum is not broken during the cooling stage.

#### 2.4.8 Cooling and storage

After the fermentation process, the product needs to be cooled in order to control the metabolic activity of the starter culture and their enzymes. Cooling of the coagulum generally commences directly after the product reaches the desired pH which is around 4.6.

### 2.5 Microbiology of the Starter Cultures

#### 2.5.1 Taxonomic information

The use of yoghurt bacteria and intestinal species of bifidobacteria and *Lb. acidophilus* in fermented milk products, containing high levels of viable organisms at the time of consumption, is dependent on the characteristics of these micro-organisms. As mentioned elsewhere, only five species of bifidobacteria (*Bif. bifidum*, *breve*, *longum*, *adolescentis* and *infantis*) and human isolates of *Lb. acidophilus* have been used for the manufacture of therapeutic fermented milk products. The differentiation characteristics of these micro-organisms have been reported by Rasic and Kurmann (1983), Mitsuoka (1984,1992), Scardovi (1986), Modler *et al.* (1990) and Ballongue (1993), and could be summarised as follows.

### 2.5.1.1 Morphology

Bifidobacteria are anaerobic, non-motile and non-spore forming micro-organisms. The cells are invariably Gram-positive rods (0.5-1.3 by 1.5-8.0  $\mu\text{m}$ ). These rods, with an irregular outer wall, are usually concave and their extremities generally swollen to form 'lumps'. It is, however, not unusual to encounter more rounded shapes as well as very long or short bacilli of varying widths. They may also appear as V, Y, X or club shapes depending on the medium constituents used to grow them. It is believed that in adverse growth conditions, the cell morphology exhibits changes resulting in a cell with greater branching. In a medium deficient in  $\beta$ -methyl-D-glucosamine the bifidus cells take on a more 'branched shape'. By adding certain amino-acids (*e.g.* serine, alanine, aspartic acid) highly branched bifidus cells become curved rods (Glick *et al.*, 1960).

The cell morphology of bifidobacteria species grown anaerobically in stabs of trypticase-phytone-yeast extract medium showed that some of them had distinctive cellular shapes. For example, *Bif. Bifidum* forms groups of 'amphora-like' cells, *Bif. breve* cells are specific epithet, thinnest and shortest among bifidobacteria and *Bif. longum* are very elongated, relatively thin cells with slightly irregular contours and rare branching.

*Lb. acidophilus*, as most of the facultative anaerobic lactobacillus species, are non-sporing, and Gram-positive cells. They are rods with rounded ends, generally 0.6-0.9 by 1.5-6  $\mu\text{m}$ , occurring singly, in pairs or in short chains (Moro, 1900; Hansen and Mocquot, 1970). In contrast, the cells size of *Lb. delbrueckii* ssp. *bulgaricus* grown in milk ranges between 0.8-1.0 by 4-6  $\mu\text{m}$ , and the cells occur mainly singly or in pairs. Culture media and temperature affect the morphology of *Lb. delbrueckii* ssp. *bulgaricus*. For example, growth in milk at 22 °C results in long filaments (in some cases up to 500  $\mu\text{m}$ ), and the cells become irregular (Rasic and Kurmann, 1978).



*Str. thermophilus* cells are non-motile, spherical or ovoid shape, 0.7 to 0.9  $\mu\text{m}$  in diameter, and occurring in pairs to long chains. The morphology of *Str. thermophilus* is also affected by the nutrients in the growth media and temperature. Growth in milk at 45 °C it forms short chains, whilst at 30 °C many strains appear as diplococci. It may form long chains at higher temperatures (~45 °C) than at lower temperatures (~30 °C) (Frank, 1964).

### 2.5.1.2 Cell wall structure

A summary of cell wall and deoxyribo-nucleic acid (DNA) contents of bifidobacteria species and other lactic acid bacteria used in this study are shown in Table 2.5. The principal component of the cell wall of bifidobacteria species is peptidoglycan or murein. This is a macro-molecule that consists of linear polysaccharide chains (glucose, galactose and rhamnose) which are linked with each other by tetrapeptide bridges associated with peptides (Ballongue, 1993).

**Table 2.5** Cell wall composition of some selected species of lactic acid bacteria.

Species	Type of peptidoglycan or murein	DNA (G+C) mol %	Polysaccharides		
			Glucose	Galactose	Rhamnose
<i>Bif. adolescentis</i>	Lys or Orn-D-Asp	58.9 <sup>a</sup>	+	+	-
<i>Bif. bifidum</i>	Orn or Lys-D-Ser-D-Asp	60.8 <sup>a</sup>	+	+	+
<i>Bif. breve</i>	Lys- Gly	58.4 <sup>a</sup>	+	+	+
<i>Bif. infantis</i>	Orn or Lys-Ser-Ala-Thr-Ala	60.5 <sup>a</sup>	+	+	+
<i>Bif. longum</i>	Orn or Lys-Ser-Ala-Thr-Ala	60.8 <sup>a</sup>	+	+	+
<i>Lb. acidophilus</i>	Lys-D-Asp	34-37	N	N	N
<i>Lb. delbrueckii</i> ssp. <i>bulgaricus</i>	Lys-D-Asp	49-51	N	N	N
<i>Str. thermophilus</i>	Lys-Ala <sub>2-3</sub>	37-40	N	N	N

<sup>a</sup> Average values.

N No data available.

Adapted from Tamime *et al.* (1995) and Sgorbati *et al.* (1995).

### 2.5.1.3 Carbohydrate utilisation

Table 2.6 illustrates the carbohydrates utilisation of bifidobacteria species, *Lb. acidophilus*, *Lb. delbrueckii* ssp. *bulgaricus* and *Str. thermophilus*. It is evident that among the *Bifidobacterium* spp., *Bif. adolescentis* can utilise a wider range of carbohydrates, followed by *Bif. breve*, *infantis*, *longum* and *bifidum*, respectively. However, all these five species can grow in milk because they are able to ferment lactose.

*Lb. acidophilus* is able to ferment maltose, sacharose, cellobiose, threhalose, mannose, salicin, glucose, fructose, galactose and lactose, whereas *Lb. delbrueckii* ssp. *bulgaricus* is able to ferment fewer carbohydrates (*i.e.* glucose, fructose, galactose and lactose). *Str. thermophilus* is able to ferment glucose, fructose, lactose, saccharose, but is not able to ferment maltose.

### 2.5.1.4 Deoxyribonucleic acid (DNA) homology

The guanine plus cytosine (G+C%) molecular percentage of the cultures used in this study is shown in Table 2.5. The G+C percentage for the bifidobacteria species ranges between 58.4 to 60.8, and for *Lb. acidophilus* ranges between 34 to 37. With respect to the relatedness of DNA homology among the bifidobacteria of human origin, two species are closely related, and these are *Bif. infantis* and *longum* (Lauer and Kandler, 1983; Imamura *et al.*, 1990; Yacshima *et al.*, 1992).

On the other hand, *Lb. acidophilus* strains exhibit a very low degree of DNA homology with each other (Johnson *et al.*, 1980; Sarra *et al.*, 1980; Lauer *et al.*, 1980). In contrast the DNA-DNA homology among *Lb. delbrueckii* ssp. *delbrueckii*, *Lb. delbrueckii* ssp. *bulgaricus* and *Lb. delbrueckii* ssp. *lactis*, including the respective type strains, were found highly homologous among each other (Weiss *et al.*, 1983). High homology was also found between two strains of *Str. thermophilus* and two of *Streptococcus salivarius* (Kilpper-Balz *et al.*, 1982).

**Table 2.6** Carbohydrate fermentation characteristics for differentiating some selected lactic acid bacteria.

	Micro-organism <sup>a</sup>							
	1	2	3	4	5	6	7	8
Arabinose	+	-	-	-	+	-	-	-
Cellobiose	+	-	(+)	-	-	+	-	-
Fructose	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	-	V
Glucose	N	N	N	N	N	+	+	+
Gluconate	+	-	-	-	-	-	-	N
Inulin	(+)	-	(+)	(+)	-	N	N	-
Lactose	+	+	+	+	+	+	+	+
Maltose	+	-	+	+	+	+	-	-
Mannitol	(+)	-	(+)	-	-	-	-	-
Mannose	(+)	-	+	(+)	(+)	+	-	+
Melizitose	+	-	(+)	-	+	-	-	N
Melibiose	+	(+)	+	+	+	d	-	V
Raffinose	+	-	+	+	+	d	-	V
Ribose	+	-	+	+	+	-	-	V
Salicin	+	-	+	-	-	+	-	-
Sorbitol	(+)	-	(+)	-	-	-	-	-
Starch	+	-	-	-	-	N	N	N
Sucrose	+	(+)	+	+	+	+	-	+
Trehalose	(+)	-	(+)	-	-	d	-	-
Xylose	+	-	-	(+)	(+)	-	-	-

<sup>a</sup> 1→5 *Bif. adolescentis*, *bifidum*, *breve*, *infantis* and *longum*, respectively; 6, *Lb. acidophilus*; 7, *Lb. delbrueckii ssp. bulgaricus*; 8, *Str. thermophilus*.

+

 Positive reaction by 90% or more strains.

-

 Negative reaction by 90% or more strains.

(+)

 Positive reaction, but ferment slowly.

d

 11-89% of strains are positive.

V

 Reported as variable.

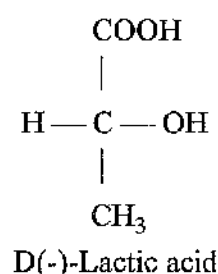
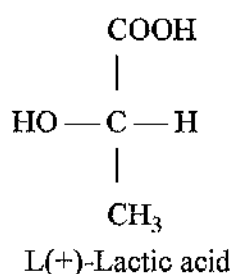
N

 No data available.

Data compiled from Tamime *et al.* (1995), Kandler and Weiss (1986) and Schleifer (1986).

## 2.6 Biochemistry and Physiology

Lactic acid bacteria, depending on the enzymatic constitution, produce L(+)-, D(-)- and DL-lactic acid. These isomers differ in the configuration of the second carbon atom.



*Lb. delbrueckii* ssp. *bulgaricus* belongs to the homofermentative group of lactic acid bacteria. It produces up to 1.7% D(-)-lactic acid in milk, whilst *Lb. acidophilus* produces between 0.3 and 1.9% DL-lactic acid. Minor quantities of carbonyl compounds, ethanol and volatile acids are also produced. *Lb. delbrueckii* ssp. *bulgaricus* is featured by its high temperature of optimum growth (40-43 °C), while *Lb. acidophilus* has an optimum growth at 37 °C and grows slowly in milk (Rasic and Kurmann, 1978).

*Str. thermophilus* is also homofermentative producing lactic acid in milk up to 0.7-0.8% of L(+)-lactic acid. Small quantities of volatile acids, such as formic, acetic, propionic, butiric, isovaleric and caproic, are also produced, as well as minor amounts of acetaldehyde, acetone and ethanol (Galesloot *et al.*, 1968; Bottazi *et al.*, 1971). Its optimum temperature of growth is between 37-40 °C. However, *Str. thermophilus* is very sensitive to antibiotics, and is readily inhibited by 0.01 IU of penicillin.

Bifidobacteria produce both acetic and lactic acids from lactose (molar ratio 3:2). The lactic acid produced is the L(+) form. These traits differentiate the bifidobacteria from *Lb. acidophilus* and *Lb. delbrueckii* ssp. *bulgaricus*, which produce DL- or D(-)-lactic acids, respectively. In addition, minor amounts of compounds like ethanol, formic and succinic acids are formed (Dellaglio *et al.*, 1992).

According to Ballongue (1993) the optimum growth temperature of bifidobacteria (human species) is between 36 and 38 °C. In contrast, bifidobacteria of animal origin grow at 41-43 °C. However, there is no growth below 20 °C and these bacteria have no thermoresistance above 46 °C. For example, *Bif. bifidum* dies at 60 °C (Rasic and Kurman, 1983). Scardovi (1986) stated that the initial optimum pH is between 6.5 and 7.0, and no growth can occur below 5.0 or above 8.0.

The bifidobacteria are strictly anaerobic micro-organisms. However, their sensitivity to oxygen differs between strains of the various species. From the industrial point of view, those species with a high tolerance to oxygen are selected for the production of fermented milk. According to Ballongue (1993) there are three types of responses observed during the switch from anaerobiosis to aerobic conditions: (a) strains which grow in presence of oxygen without the accumulation of H<sub>2</sub>O<sub>2</sub>, (b) strains with limited growth and accumulation of H<sub>2</sub>O<sub>2</sub>, and (c) no growth without the accumulation of H<sub>2</sub>O<sub>2</sub>.

### 2.6.1 Associative growth

The well known associative growth between *Lb. delbrueckii* ssp. *bulgaricus* and *Str. thermophilus* is attributed to the breakdown of milk proteins and release of amino acids by *Lb. delbrueckii* ssp. *bulgaricus* which stimulates *Str. thermophilus*. The later organism produces formic acid and carbon dioxide which stimulates *Lb. delbrueckii* ssp. *bulgaricus* (Marshall and Tamime, 1997). It is also important to note that both of these bacteria grow perfectly in milk, and this property is utilised to prepare the so-called bio-fermented milk products.

Most of the strains of bifidobacteria used in the manufacture of fermented milks grow poorly in milk without additives. Ideally the strains selected should either acidify milk to below pH 5 within 24 h at 37 °C when grown alone, (and when inoculated at a level not less than 10<sup>8</sup> cfu ml<sup>-1</sup>) or when grown in conjunction with other lactic acid bacteria such as *Lb. acidophilus* or *Str. thermophilus*. *Lb. acidophilus* seems to grow more easily in the presence of bifidobacteria rather than other organisms.

Bifidobacteria seem to grow more rapidly when *Lb. acidophilus* is present, suggesting the existence of symbiotic relationship between these two micro-organisms (Hunger and Peitersen, 1992).

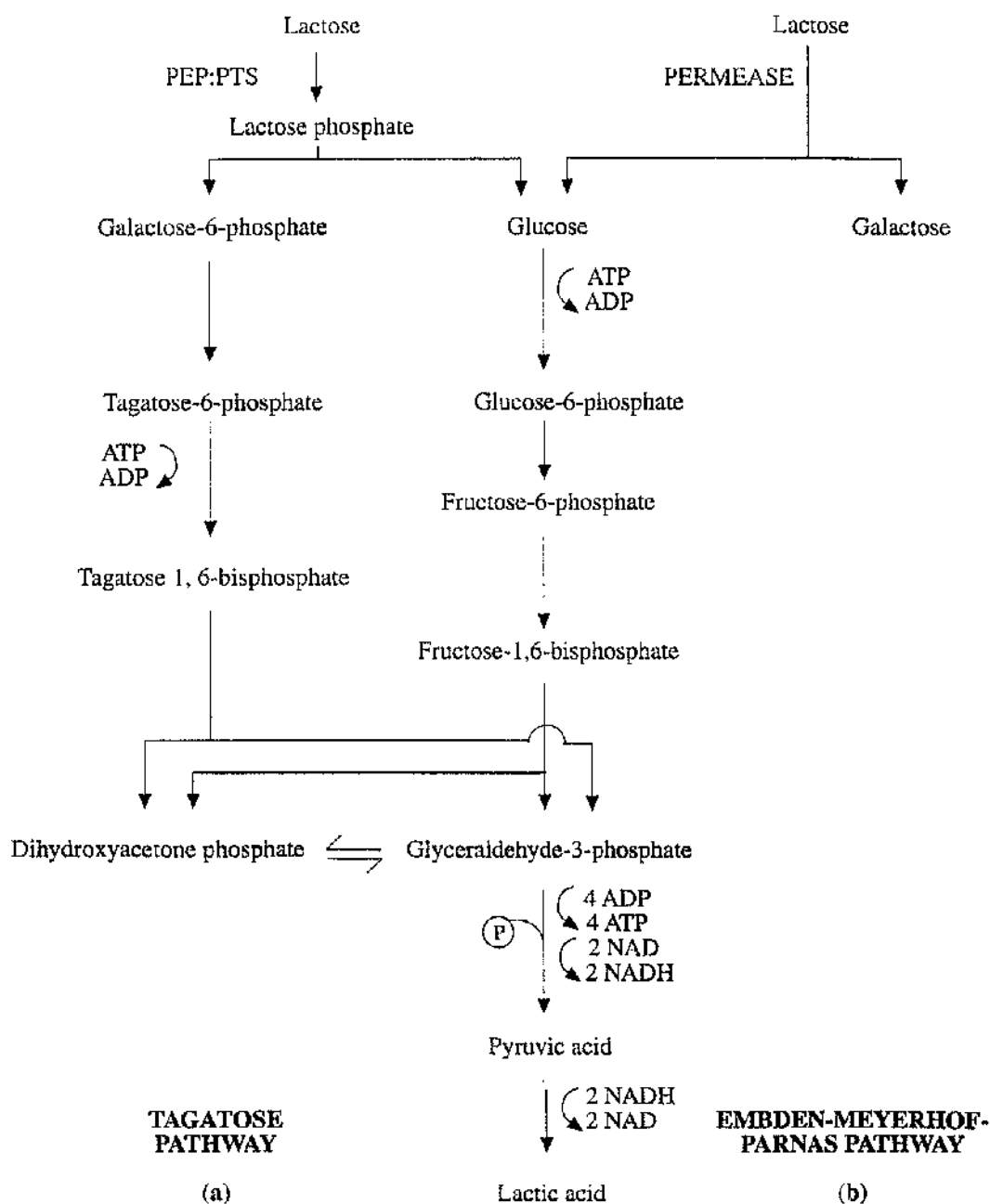
Cheng and Nasajawa (1983) stated that studies on the associative growth between lactobacilli and bifidobacteria in milk indicated a stimulation of the bifidobacteria due to proteolytic enzymes and aminopeptidase of the lactobacilli. A summary of different combination where bifidobacteria could be used to manufacture different fermented milks is shown below (Driessen and de Boer, 1989):

- Yoghurt bacteria combined with *Bifidobacterium* spp.
- One of the yoghurt bacteria (*Str. thermophilus*) combined with *Bifidobacterium* spp.
- One of the yoghurt bacteria combined with *Bifidobacterium* spp. and *Lb. acidophilus*.
- *Bifidobacterium* spp. and *Lactobacillus* spp. without the yoghurt micro-flora.

### 2.6.2 Sugar metabolism

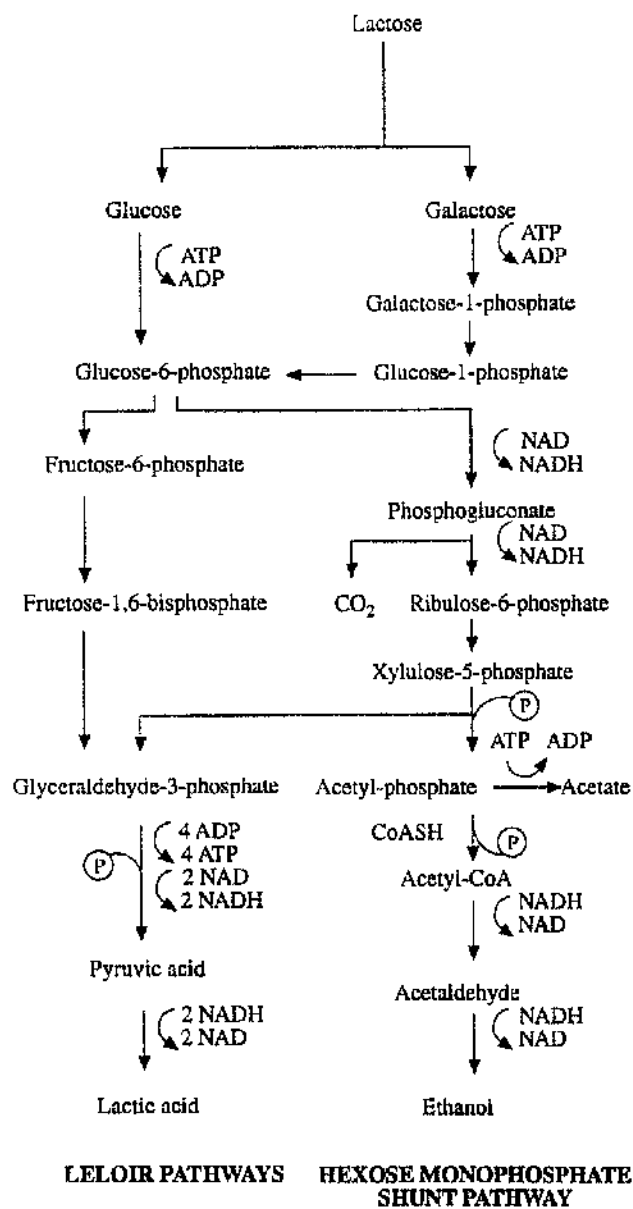
Carbohydrates are metabolised by lactic acid bacteria either through homofermentative or heterofermentative metabolic pathways. *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. acidophilus* and *Str. thermophilus* ferment lactose homofermentatively via the Emden-Meyerhof-Parnas (EMP) pathway for glucose catabolism. However, some strains of *Lb. acidophilus* differ from the other organisms mentioned, in that both glucose and galactose (galactose-6-phosphate) parts of the lactose molecule are catabolised at the same time. The former via EMP pathway and the latter via the Tagatose pathway. Figure 2.3 illustrates how lactose is phosphorylated by phosphoenolpyruvate (PEP) during translocation by the PEP-dependent phosphotransferase system (PTS) (Mc Kay *et al.*, 1969). The products of the reaction are glucose and galactose-6-phosphate. The glucose is then catabolised to pyruvate and the galactose is excreted from the cell (Marshall and Tamime, 1997). Once the glucose has been utilised, *Str. thermophilus* and *Lb. acidophilus* will utilise galactose via Leloir pathway (Figure 2.4).

**Figure 2.3** Homolactic fermentation of lactose after translocation, (a) by a PEP:PTS mechanism, and (b) by a Permease.



After Marshall and Tamime (1997).

**Figure 2.4** Galactose metabolism by the Leloir pathway and heterolactate fermentation by the hexose monophosphate shunt.



After Marshall and Tamime (1997).



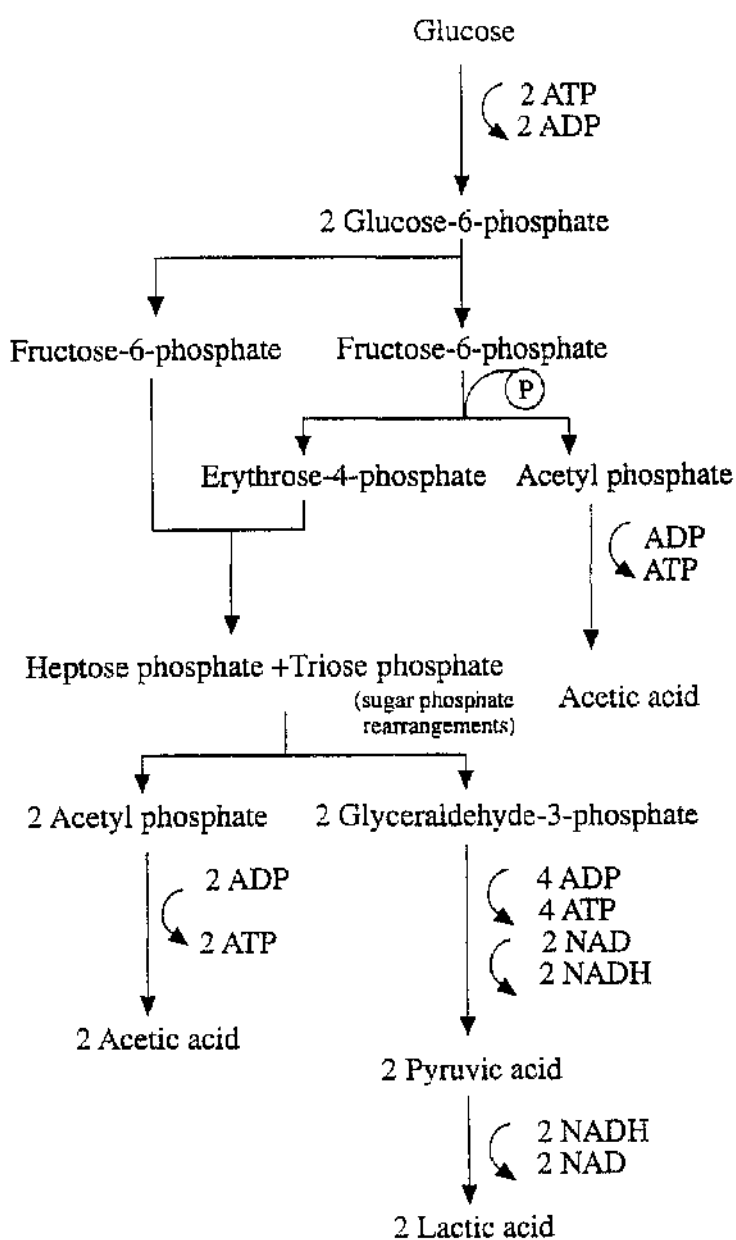
The bifidobacteria metabolises the lactose and other sugars in a different way. Hexoses are degraded exclusively and specifically by the fructose-6-phosphate pathway described by Scardovi and Trovati (1965). Aldolase and glucose-6-phosphate dehydrogenase are absent, whereas fructose-6-phosphate phosphoketolase (F<sub>6</sub>PPK) is present. A summary of the reactions involved is shown in Figure 2.5. The fermentation of two moles of glucose yields to three moles of acetate and two moles of lactate.

### 2.6.3 Nitrogen metabolism

The majority of lactic acid bacteria require or are stimulated by amino acids, and to grow in milk successfully depends on the milk protein degradation by extracellular proteinases and peptidases (Marshall and Tamime, 1997). *Str. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus* possess a number of peptidases. Stefanitsi *et al.* (1994) reported that *Lb. delbrueckii* ssp. *bulgaricus* had two cell-wall-associated proteinases whose activities were enhanced when the cells were grown in milk, and they were able to hydrolyse casein.

Most strains of bifidobacteria are able to use ammonium salts as their source of nitrogen (Hassinen *et al.*, 1951). However, some strains like, *Bifidobacterium suis* and *Bifidobacterium cuniculi* develop only in the presence of organic nitrogen (Ballongue, 1993). *In vitro* and in the absence of any organic source of nitrogen, bifidobacteria may synthesise large amounts of amino acids. *Bif. bifidum*, for example, produces alanine, valine and threonine (Matteuzzi *et al.*, 1978). Tamime *et al.* (1995) reported that some strains of bifidobacteria lack sufficient proteolytic activity for growth in milk. Therefore, milk needs to be enriched with casein hydrolysate (Klaver *et al.*, 1993) or yeast extract (Roy *et al.*, 1990).

**Figure 2.5** Sequence of reactions of hexose fermentation by *Bifidobacterium* spp.



After Marshall and Tamime (1997).

#### 2.6.4 Growth factors

Lactic acid bacteria are widely distributed in nature, but their nutritional requirements are complex. These requirements dictate the natural habitat of these organisms. For example, growth of lactic acid bacteria is rarely observed in water, but common in milk and milk products (Marshall and Law, 1984). There are several growth factors which can promote the growth of lactic acid bacteria, and they can be summarised as follows: carbohydrates, proteins, oligosaccharides, salts, minerals, vitamins and in some cases carbon dioxide. Table 2.7 shows some of the growth requirements for lactobacilli species and *Str. thermophilus*. Given the importance of growth factors of bifidobacteria species, the rest of this section discuss in more detail the so-called 'bifidogenic factors'.

Many species of *Bifidobacterium* are unable to grow properly in a medium containing minimum base requirements. In order to enhance their growth, complex biological substances are required such as bovine casein digestate, lactoserum of bovine milk, yeast extract and others (Poch and Bezkorovainy, 1988; Petschow and Talbott, 1990). These growth factors are known as bifidogenic or bifidus factors (Ballongue, 1993). There are three main groups of these growth factors [which are species dependent (Modler *et al.*, 1990)], the BB (BF<sub>1</sub>, BF<sub>2</sub> and glycoproteins), BI and BL factors. In fact the *Bif. Bifidum* species can be divided into two variants according to their origin, *i.e.* BBa and BBb. The former refers to *Bif. bifidum* found in adult humans and the latter is referred to *Bif. Bifidum* isolated from infants.

- Bifidus factor I or BF<sub>1</sub>- this is found in milk and colostrum and in the form of gynolactose which is active particularly on *Bif. bifidum* variant BBb (Ballongue, 1993).
- Bifidus factor II or BF<sub>2</sub>- this consists of nonglycosylated peptides obtained by the action of a protease on casein, and according to Raynaud (1959) such factors stimulate the strain *Bif. bifidum* variant BBa.

Table 2.7 Growth requirements of some selected bacteria.

	<i>Str. thermophilus</i>	<i>Lactobacillus</i> spp.
<i>Amino-acids</i>		
Lysine	+	+
Leucine	+	+
Histidine	+	+
Valine	+	+
Cysteine	+	S
Aspartate	+	+
Glutamate	±	+
Isoleucine	±	+
Tyrosine	±	+
Methionine	±	+
<i>Vitamins</i>		
Vitamin B <sub>12</sub>	+	-
Biotin	+	+
Niacin	+	+
Pantothenate	+	+
Riboflavin	+	+
Thiamine	+	-
Pyridoxal	+	-
Folic acid	+	-
<i>Organic acids</i>		
Acetic	NI	NI
Oleic	NI	S
Orotic	NI	S
Formic	NI	S

+ Essential for growth.

± Essential only in some strains.

- Not required for growth.

NI Not investigated.

S Stimulatory.

After Marshall and Law (1984).

- Glycoproteins- these are isolated from human colostrum and milk lactoserum which seem to be effective for both variants (*i.e.* BBa and BBb) (Seka Assy, 1982).
- BI and BL factors- these are abundant in many plants, liver and milk extracts. The BI factor, which are proteins, stimulates the growth of *Bif. infantis*, and is destroyed by lyophilization. Whereas the BL factor (from human milk), which stimulates the growth of *Bif. longum*, is sensitive to heating and irradiation (Beerens *et al.*, 1980).

Other growth promoting factors of bifidobacteria include lactoferrin and oligosaccharides (natural and synthetic). For example, lactoferrin and its three metal complex (Fe, Cu, Zn) have a promoting effect on eight species of *Bifidobacterium*, and fructo-oligosaccharides (FOS) have a stimulant effect on the growth of bifidobacteria (Hidaka *et al.*, 1986). A source of FOS is the Jerusalem artichoke tuber (Tamime *et al.*, 1995).

#### 2.6.5 Exopolymer production

Exopolymers are polysaccharides that are produced by certain strains of lactic acid bacteria during the fermentation process. The use of starter micro-organisms, which produce exopolymers, play an important role with regard to the consistency of the fermented milk products (*i.e.* viscoelastic and/or thixotropic properties).

Some lactobacilli strains produce heteropolysaccharides which are polymers branched and can differ in composition depending on the carbohydrate on which they have grown. *Str. thermophilus* produce a polysaccharide that is composed mainly of galactose residues (Doco *et al.*, 1990). In contrast, bifidobacteria have extracellular or surfaces enzymes that catalyse the hydrolysis of complex polysaccharides: amylose, amylopectin, xylan, gum arabic (Salyers *et al.*, 1978).

## 2.7. Therapeutic Aspects of Fermented Milks

Over the last few years research work carried out on bifidobacteria and lactobacilli have increasingly demonstrated evidence to suggest that these micro-organisms, when consumed in sufficient large number, exhibit prophylactic and therapeutic properties in humans and animals (Mitsuoka, 1990; Robinson, 1991; Sandine *et al.*, 1972). For example, Yamasaki *et al.* (1982) indicated that the presence of bifidobacteria on the walls of the colon may induce cell-mediated against *Escherichia coli*, and Robinson and Samona (1992) suggested that both lactic and acetic acids produced by bifidobacteria may show specific toxicity against some species of yeast or bacteria.

Various studies insinuating different desirable features of bifidobacteria have also been published. Tamime *et al.* (1995) suggested that to get the beneficial effects from fermented milk products manufactured by using the so-called probiotic micro-organisms; some conditions are of paramount importance, and these are:

- A regular consumption at a level of 400 to 500 g of product per week.
- A number of at least  $1 \times 10^6$  cfu  $g^{-1}$  in the product at the time of consumption (Robinson, 1989).
- The species selected should be of human origin and able to survive passage through the upper region of the intestinal tract.

### 2.7.1 Nutritional composition

The chemical composition of the milk base is different from normal milk because the fat content is normally reduced and the SNF is fortified which increases the lactose and protein contents. However, the nutritive value of yoghurt and fermented milk products in the human diet is determined by the nutritive value of milk base from which it was made. Differences may occur from chemical changes of milk constituents during the fermentation stage such as : reduction of lactose content, formation of lactic acid and other organic acids, increase the free peptides content, amino acids and fatty acids content, and also changes in the vitamin content since certain strains of starter cultures are capable to synthesise some water soluble vitamins (Rasic and Kurmann, 1978).

### 2.7.2 Digestibility

During the manufacture of yoghurt and fermented milk products, the digestibility of proteins and fat is significantly increased due to the effect of lactic acid, the proteolytic activity of the starters, the homogenisation effect and lipase activity of cultures (Rasic and Kurmann, 1978). For example, the digestion process for protein in yoghurt requires half the time of the correspondent original milk base (Salji, 1989; Odet, 1990).

### 2.7.3 Lactose intolerance

Lactose intolerance in some individuals is caused by a deficiency in the intestinal tract of  $\beta$ -galactosidase resulting in the inability to digest such disaccharide. The reduction of gastrointestinal symptoms in lactose intolerant individuals are attributed to the following aspects: (a) decrease up to 30% or more of the lactose present in the milk base due to starter culture metabolism and (b) the release of  $\beta$ -galactosidase as a consequence of the fermentation process (Rasic and Kurmann, 1978; Hunger and Peitersen, 1992).

### 2.7.4 Synthesis of vitamins

Shahani and Chandan (1979) reported that B-vitamins content of fermented milk products is affected by the amount and type of micro-organisms in the starter. While many lactic acid bacteria require B-vitamins for growth, several other micro-organisms are capable of synthesising certain vitamins. The same authors revealed that fermented milks certainly contain higher folic acid, niacin, biotin, pantothenic acid, B<sub>6</sub> and B<sub>12</sub> than the milk from which were made.

Teraguchi *et al.* (1984) and Deguchi *et al.* (1985) stated that some strains of bifidobacteria of human origin require thiamine (B<sub>1</sub>), pyridoxine (B<sub>6</sub>), folic acid (B<sub>9</sub>), cyanocobalamin (B<sub>12</sub>) and nicotinic acid (PP) for their growth. The latter authors reported that certain vitamins of group B, with the exception of riboflavin (B<sub>2</sub>), are

synthesised by most of the strains examined, and they indicated that the production of vitamins B<sub>2</sub> and B<sub>6</sub> by *Bif. longum* was exceptional. Meanwhile, *Bif. breve* and *Bif. infantis* produce high level of vitamins PP and H, respectively. Table 2.8 illustrates the vitamin production and requirement of the bifidobacteria from human origin.

**Table 2.8** Vitamin production and requirement of some strains of bifidobacteria.

	<i>Bif. breve</i>	<i>Bif. infantis</i>	<i>Bif. longum</i>	<i>Bif. bifidum</i>	<i>Bif. adolescentis</i>
Thiamin (B <sub>1</sub> )	+	+++	+	+++	+
Riboflavin (B <sub>2</sub> )	+	+	+++	++ (*)	+
Pyridoxine (B <sub>6</sub> )	++	++	+++	+	++ (*)
Folic acid (B <sub>9</sub> )	+	+++	+	++ (*)	+
Cobalamin (B <sub>12</sub> )	+	++	+++	+	+
Nicotinic Ac.(PP)	+++	+++	+	+++ (*)	+
Biotin (H)	++	+++	++	++	++

+, ++, +++ Capability of production in ascendant order.

(\*) Required for growth.

Adapted from Ballongue (1993) and Scardovi (1986).

### 2.7.5 Antagonistic effects

The observation that lactic acid bacteria, including yoghurt bacteria, *Lb. acidophilus* and bifidobacteria, have antagonistic effects against pathogenic bacteria (e.g. *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella* spp. and *Clostridium* spp.) is attributed to the fermentative conversion of lactose to organic acids, mainly lactic and acetic acids. Thus, reduction of the pH of milk from ~6.8 to <4.6 leads to increase the shelf-life and safety of the products.

According to Tamime and Marshall (1997) the inhibitory activity is controlled by the dissociation constant ( $pK_a$ ) and the acid concentration at a given pH. Thus, an organic acid of high  $pK_a$  value has more undissociated acid and has a stronger antimicrobial activity. For example, the  $pK_a$  of lactic acid is 3.85 whilst of acetic acid is 4.73 (Gould, 1991), and therefore the later possess a stronger antagonistic effect. Mital and Garg (1995) reported that the antagonistic effect of *Lb. acidophilus* and other lactic acid bacteria was possibly due to competition for nutrients, adhesion sites,



formation of metabolites (*i.e.* organic acids), production of hydrogen peroxid and bacteriocins.

Bifidobacteria are claimed to possess a broad spectrum of anti-microbial activity. For example, Gibson and Wang (1994) reported that bifidobacteria could excrete an anti-microbial substance which was able to exert inhibitory effect to species such as *Escherichia coli* and *Clostridium perfringens*, *Salmonella* spp., *Listeria* spp., *Campylobacter* spp., and *Shigella* spp., as well as *Vibrio cholerae*.

### 2.7.6 Bacteriocins

Bacteriocins are characterised as heterogeneous group of antimicrobial substances with respect to producing bacteria, antimicrobial spectrum, mode of action and chemical properties (Daeschel, 1989). According to Tamime and Marshall (1997) there are around 70 different types of bacteriocins that have been identified and produced by lactic acid bacteria. However, Juillard *et al.* (1987) have suggested the following definition for bacteriocins:

- They generally have narrow range of action.
- Part of the molecule is a peptide and therefore they are sensitive to proteases.
- They are thermostable.

One of the best characterised bacteriocin is 'Nisin' which is produced by *Lactococcus lactis* ssp. *lactis* and possess inhibitory action against the Gram positive pathogens such as *Listeria*, *Clostridium*, *Staphylococcus*, *Bacillus*, *Enterococcus* and *Salmonella* spp. (Dodd and Gasson, 1994). *Bif. bifidum* produce an antibacterial substance named 'Bifidin' which was heat stable and showed antibacterial activity against *Micrococcus flavus* and *Escherichia coli* (Anand *et al.*, 1985). *Lb. acidophilus* produce some inhibitory substances such as 'lactocidin', 'acidophilin' and 'acidolin' (Vincent *et al.*, 1959; Vakil and Shahani, 1965; Hamdan and Mikolajcik, 1974).

### 2.7.7 Bile salt tolerance and cholesterol 'assimilation'

It has been suggested that cholesterol lowering may be effected by an ability of certain bacteria to assimilate cholesterol. This particular ability occurs in the presence of bile salts under anaerobic conditions (Gilliland *et al.*, 1985; Walker and Gilliland, 1993). Another possible mechanism of cholesterol-lowering effect has been attributed to the ability of some bacteria to deconjugate bile salts which can cause more intestine bacterial inhibition than the conjugated forms, and they are less efficient in aiding lipid absorption from the intestinal tract as compared to conjugated acids. This phenomenon could influence the reduction in absorption of cholesterol from the intestine.

Some strains of *Lb. acidophilus* and bifidobacteria are considered to possess this innate ability. In addition, bifidobacteria are considered capable of breaking down conjugated bile acids to free bile acids (*i.e.* deconjugate) (Laroia and Martin, 1990; Gilliland and Speck, 1977). Recently, Tahri *et al.* (1995) reported that growing bifidobacteria cells are able to remove cholesterol both by precipitation and assimilation.

### 2.7.8 Miscellaneous

Several other therapeutic properties such as stimulation of the immune system, antitumor and anti-carcinogenic properties, have been attributed to the so-called probiotic-starter cultures. For example, the degradation of nitrosamines (compounds which include those considered to be potentially carcinogenic) by the bifidobacteria could play an important role in reducing the cancer of colon (Tamime *et al.*, 1995).

De Simone *et al.* (1988) reported that natural killer cells were increased from 103 to 147 units when they administered dietary lactobacilli and plain yoghurt to healthy persons; skimmed milk was used as a control. Usually *Lb. acidophilus* and bifidobacteria are regarded to possess these novel properties; however, special care should be taken with respect to the selection of the strains, because it was found that great differences occur between species of the same genera (Bereda *et al.*, 1991).

## 2.8 Health and Future Developments of Fermented Milk Products

Nowadays the word probiotic is widely used to refer to viable bacteria, cultured dairy products or food supplements containing viable lactic acid bacteria (Salminen *et al.*, 1993). Morelli (1991) defined probiotics as: "organisms and substances which contribute to intestinal microbial balance". However, both definitions lead to the same target, *i.e.* to have beneficial health effects in the host by improving the integrity of the intestinal micro-flora.

*Lb. acidophilus* as well as some strains of bifidobacteria are considered to have the above named beneficial health effects. A summary of these effects due to consumption of fermented dairy products containing an abundance of 'bio' culture could be summarised as follows:

- Improvement of lactose intolerance;
- Influence on pathogenic micro-organisms (*i.e.* antimicrobial properties);
- Reduction of cholesterol level;
- Tumour-inhibiting effect;
- Activation of immune system;
- Inactivation of toxic compounds.

It is understood that some of the above aspects are not well established, because of relevant *in vivo* experiments on humans are not possible. Tamime *et al.* (1995) suggested that to get beneficial human health effects, the micro-organism selected should comply with the following requirements:

- Non pathogenic and of human origin;
- Capable to survive and grow in the intestinal tract;
- Be able to attach to the gut epithelial cells, and
- Be viable, active and abundant in the product at the time of consumption.

The same authors addressed some very important aspects to take into account for a possible future development in this field, these are:

- Selection of suitable strains, capable of fermenting milk within a reasonable time;

- Wider application of bifidobacteria in the dairy industry;
- Possible genetic manipulation in order to enhance their growth in milk;
- Improvement in the design of experiments involving human volunteers, and
- Greater involvement of the medical profession in human experiments.

## 2.9 Conclusion

It is well established that the constant growing consumption of these products in most countries of the world, including developing countries is attributed to the health benefits associated with lactic acid bacteria. Despite several studies achieved on dairy products fermented by bifidobacteria, and/or in combination with yoghurt cultures (*Str. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus*) or *Str. thermophilus* alone and *Lb. acidophilus*, there is still need for further research to characterise the quality of fermented milk made with mixed cultures containing bifidobacteria species.

Taking into consideration the controversial behaviour of bifidobacteria, and the wide range of possible combinations of products fermented by bifidobacteria in combination with other lactic acid bacteria, this work was undertaken to evaluate the quality of set-type fermented milks using: either five single strains of *Bifidobacterium* spp. or seven commercial mixed starter cultures containing bifidobacteria. A yoghurt starter culture was used as a control.

## **CHAPTER THREE:**

# **EXPERIMENTAL MATERIALS AND METHODS**

## **CHAPTER THREE: EXPERIMENTAL MATERIALS AND METHODS**

### **3.1 Raw Materials**

#### **3.1.1 Skimmed milk powder (SMP)**

Medium heat skimmed milk powder of the required good quality was used throughout this study. The powder was obtained from A.N. Garret & Co. Ltd., Edinburgh, UK, in 25 kg bags and then stored in a cold and dry place.

#### **3.1.2 Anhydrous milk fat (AMF)**

Anhydrous milk fat obtained from Bodfari Producers Ltd., Chester, UK was used to standardise the milk base. The AMF was received in packages of 25 kg and stored at - 40 °C.

#### **3.1.3 Milk protein concentrate powder (MPC)**

In order to enrich the protein content in the milk base, milk protein concentrate powder obtained from DMV International, Veghel, The Netherlands, was used throughout this experimental work. The MPC powder was received in a 20 kg bag and maintained in a cold and dry place.

#### **3.1.4 Growth additives**

To enhance the growth of *Bifidobacterium* species, two different oligisaccharides were used as growth promoters (*i.e.* Jerusalem artichoke flour and Raftiline). Jerusalem artichoke whole tuber flour was obtained from Zumbro Natural Ingredients, Inc., Hayfield, Minnesota, USA, and Raftiline (inuline) which extracted from Chicory Root was obtained from Siber Hegner Limited, Kent, UK.

### 3.1.5 Starter cultures

#### 3.1.5.1 Single strains of *Bifidobacterium* spp.

Pure, freeze-dried single strains of *Bifidobacterium* spp., from the National Collection of Food Bacteria (NCFB), Reading, UK, were used to ferment the milk base. The starter cultures were received in sealed under vacuum glass ampoules. The specific micro-organisms were: *Bifidobacterium adolescentis* NCFB 2230, *Bifidobacterium bifidum* NCFB 2715, *Bifidobacterium breve* NCFB 2258, *Bifidobacterium infantis* NCFB 2205 and *Bifidobacterium longum* NCFB 2259.

Pure, freeze-dried commercial single strains of *Bifidobacterium* spp. from three different suppliers were also used to ferment the milk base. The specific micro-organisms were as follows: *Bif. longum* BB46 and *Bif. bifidum* Bb11 were obtained from Hansen's Laboratory, Ltd., Reading, UK; *Bif. longum* BBL, *Bif. infantis* BBI 410 and *Bif. longum* BL were obtained from Texel-Rhône-Poulenc, Cheshire, UK; *Bif. longum* 2 and *Bif. longum* 913 were obtained from Laboratorium "VISBY" Tønder, Denmark.

#### 3.1.5.2 Commercial mixed starter cultures

Eight commercial mixed starter cultures designed for direct-vat-inoculation (DVI) were used, and the micro-organisms present in each individual starter are shown in Table 3.1. The starters were obtained from four different suppliers (Chr. Hansen's Laboratory Ltd., Reading, UK.; Texel-Rhône-Poulenc, Cheshire, UK; Laboratorium "VISBY" Tønder, Denmark, and Systems Bio-Industries (SBI) Ltd., Newbury, Berks, UK). The starters were stored at - 40 °C until required.

## 3.2 Processing Equipment and Utensils

### 3.2.1 Batch pasteuriser

A cylindrical batch pasteuriser of stainless steel, 150 litres of capacity (T. Giusti & Son Ltd., London, UK) was used to prepare and pre-heat the milk base. The tank was fitted with steam injection system, thermometer and agitator for an appropriate recombination of the raw materials.

**Table 3.1** The micro-organisms used in commercial mixed starter cultures.

Code	Supplier	Type	Micro-organism			
			<i>Lb. acidophilus</i>	<i>Bifidobacterium</i> spp. <sup>a</sup>	<i>Str. thermophilus</i>	<i>Lb. bulgaricus</i>
AB	Hansen	Freeze dried	+	+	-	-
AC/BL	Texel	Freeze dried	+	<i>Bif. longum and infantis</i>	-	-
DV B-100	SBI	Freeze dried	+	<i>Bif. bifidum</i>	+	-
MSK 2	Visby	Deep frozen	+	<i>Bif. longum</i>	+	-
ABT-1	Hansen	Freeze dried	+	+	+	-
ABT-3	Hansen	Freeze dried	+	+	+	-
MSK B2	Visby	Freeze dried	+	+	+	-
MY 087	Texel	Freeze dried	-	-	+	+

<sup>a</sup> Denotes that the starter culture consists of more than one species of bifidobacteria not identified by the supplier; however, the cultures that have been identified by the supplier are shown in this column.

+ Denotes presence of the micro-organism in the starter culture.

- Denotes absence of the micro-organism in the starter culture.

### 3.2.2 Homogeniser

A homogeniser model Lab 4746/72 (Rannie Machine Works, Ltd., DK-2620 Albertslund, Denmark) was used to homogenise the milk base.



### **3.2.3 Pasteurisation containers**

Cylindrical containers of stainless steel of 12 litres of capacity were used for pasteurising the milk base. The pasteurisation process was carried out by using a water bath heated by steam injection.

### **3.2.4 Thermometer**

A portable digital thermometer Testo 900 (Testoterm Ltd., Hampshire, UK) was used to measure the temperature values during the different stages of the process.

### **3.2.5 Hydrogen ion meter**

A portable pH meter model 'Check Mate 90' (Mettler Toledo Ltd., Essex, UK) fitted with a combined glass electrode was used to measure the pH values in the milk base and in the products. The equipment was calibrated by using standard buffer solutions of pHs 7 and 4 (BDH Chemicals Ltd., Poole, UK) prior measuring the pH of the samples.

### **3.2.6 Incubation cabinet**

A thermostatically controlled incubator type P-33 A-18 (LEEC Electrical Engineering Ltd., Nottingham, UK) was used to ferment the milk base.

### **3.2.7 Plastic containers**

Two sizes of white cup (150 and 80 ml) supplied by MONO Containers Ltd., Middlesex, UK were used for the production of set-type fermented milk. The cups were suitable for "press-on" lids.

### **3.3 Production of set-type fermented milk**

The set-type fermented milk products were produced following the stages shown in Figure 2.2. The skimmed milk powder and milk protein concentrate [ $\sim 8.05$  and  $\sim 128$  g of product  $\text{kg}^{-1}$  respectively] were reconstituted at  $40^\circ\text{C}$  to  $\sim 13.0$  g total solids  $100\text{g}^{-1}$ , and then heated to  $60^\circ\text{C}$ . The anhydrous milk fat was melted in a water bath at  $65^\circ\text{C}$  and added at a rate of  $1.5\text{g } 100\text{g}^{-1}$  to the milk base. The mix was then homogenised at a pressure of 17 MPa, and divided in equal portions of  $\sim 11$  kg. Each portion was heated at  $90^\circ\text{C}$  for 5 min using a water bath provided with steam injection system, and cooled to  $45^\circ\text{C}$  by means of mains running water.

The processed milk bases were then inoculated with the different starter cultures at a rate of 2g per 10 kg and dispensed into the plastic cups (150 and 80 ml) and incubated at  $40^\circ\text{C}$  until the pH was  $\sim 4.60$ . The fermented milks were then transferred to a cold store at  $\sim 4^\circ\text{C}$  where the products were stored until they were required for the different assessments.

### **3.4 Chemical and Microbiological Analysis of Skimmed Milk Powder (SMP) and Milk Protein Concentrate (MPC)**

#### **3.4.1 Determination of fat**

Fat contents of SMP and MPC were determined according to the methods of the International Dairy Federation (IDF) (1987b) which is based on the Rose-Gottlieb analytical method.

#### **3.4.2 Determination of total nitrogen**

The improved Kjeldahl method of the British Standards Institution (BSI, 1968) was used to determine the total nitrogen content of SMP and MPC. Kjeldahl catalyst tablets supplied by BDH Chemicals Ltd. were used instead of mercuric oxide. A standard solution of 1% Boric acid (containing indicator) was used as a receiver

solution. After the distillation process the ammonia was titrated with a standard solution of 0.02 N of hydrochloric acid (HCl) using a Kjeltac Auto 1030 analyser (Tecator AB, Box 70, Hoganas, Sweden).

### **3.4.3 Determination of total solids**

The IDF (1993) method was used to determine the water content of SMP and MPC. Approximately 3 g of sample was weighed on a AE balance (Mettler Instruments Ltd., Buckinghamshire, UK) and dried in a oven at 102 °C to a constant weight (Galenkamp Express, Loughborough, Leicestershire, UK).

### **3.4.4 Determination of ash**

The ash content of SMP and MPC was determined according to the Association of Analytical Chemists (AOAC, 1990). It was achieved by ignition of the sample at 550 °C in a muffle furnace (Baird and Tatlock, London, UK).

### **3.4.5 Determination of the titratable acidity**

The titratable acidity of SMP was determined according to the method specified by the American Milk Dry Institute (ADMI, 1983).

### **3.4.6 Determination of the heat number**

The heat number of SMP was determined according to the method described by the International Dairy Federation (IDF, 1982b).

### **3.4.7 Determination of the solubility index**

The solubility index of the SMP was determined according to the method of the American Dry Milk Institute (ADMI, 1983).

### **3.4.8 Scorched particles**

The scorched particles content in SMP and MPC was determined according to the method described by the American Dry Milk Institute (ADMI, 1983).

### **3.4.9 Antibiotics residue**

The test described by Crawford and Galloway (1964) was used for the detection of antibiotics and other inhibitors in the SMP and MPC. This test detects the presence of antibiotic and other inhibitors residues at a level of 0.02 International Units (IU) of penicillium ml<sup>-1</sup>. The test organism was *Bacillus stearothermophilus* var. *calidolactis*, and the disc assay was incubated at 55 °C for 2 ½ hours.

### **3.4.10 Total viable count**

Plate Count Agar CM 325 (Oxoid Ltd., Basingstoke, UK) was used to determine the colony count. The pour plate method described by the American Dry Milk Institute, and the poured plates were incubated at 30 °C for 72 hours.

### **3.4.11 Thermoduric count**

Plate Count Agar CM 325 (Oxoid Ltd.) was utilised to determine the count of thermoduric bacteria, following the method described by Harrigan and McCance (1976). The poured plates were incubated at 55 °C for 48 hours.

### **3.4.12 Enterobacteria count**

The enumeration of enterobacteria was carried out by the method described by the IDF (1985). The test media was violet red bile lactose CM7 (Oxoid Ltd.). The plates were incubated at 30 °C for 48 hours. The confirmation test using green brilliant broth was no necessary due to the high microbiological quality of the products tested.

### **3.4.13 Yeasts and moulds count**

The enumeration of yeasts and moulds was achieved by the method described by IDF (1990). Yeast extract-dextrose-chloramphenicol-agar medium was used. The chloramphenicol content was calculated in order to obtain a final concentration of 1  $\mu\text{g } 100\text{g}^{-1}$  in the medium.

## **3.5 Analysis of Anhydrous Milk Fat (AMF)**

### **3.5.1 Determination of fat and moisture**

The fat content of AMF was determined by the method described in section 3.4.1. The moisture content was determined by the Karl Fischer method described by the IDF (1988a).

### **3.5.2 Determination of the peroxide value**

The IDF (1991a) method was used to determine the peroxide value of the anhydrous milk fat.

### **3.5.3 Microbiological analysis**

Total viable, thermotolerant, enterobacteria, and yeasts and moulds counts were determined according to the methods described in sections 3.4.10., 3.4.11., 3.4.12. and 3.4.13., respectively.

### **3.5.4 Lipolytic count**

The enumeration of lipolytic micro-organisms was determined according to the method described by Harrigan and McCance (1976). Tributyrin agar PM4 (Oxoid Ltd.) was used as a medium and the plates were incubated at 30 °C for 36 hours.

### 3.6 Enumeration of the Micro-organisms in the Starter Cultures

The enumeration of the lactic acid bacteria in the starter cultures were carried out by the pour plate method (Harrigan and McCance, 1976), and using different selective media depending on the type of micro-organism present in each individual starter. All micro-organisms were enumerated under aseptic conditions. Blends of 10 g of starter in 90 ml of Ringer diluent (Quarter-strength Ringer Solution BR52, Oxoid Ltd.), and further serial dilutions (*i.e.* 1ml of the blend into 9 ml of diluent) were prepared. The dilution levels were determined according to the manufacturer's specifications.

#### 3.6.1 Media preparation

Three types of mixed starter cultures were used (see Table 3.1), for example: (a) *Bifidobacterium* spp. is used only with *Lb. acidophilus*, (b) *Bifidobacterium* spp. is used with *Lb. acidophilus* and *Str. thermophilus*, and (c) the yoghurt starter containing *Str. thermophilus* and *Lb. bulgaricus*. The media used were:

- (a) For *Bifidobacterium* spp., MRS agar CM 361 (Oxoid Ltd.) with the addition of 0.05g 100g<sup>-1</sup> of Cysteine C-775 (Sigma Ltd.) and 5ml 100ml<sup>-1</sup> of NNL solution, which consisted of Lithium Chloride (BDH Chemicals Ltd.), Nalidixic acid N-8878 (Sigma Ltd.) and Neomycin Sulphate N-1976 (Sigma Ltd.). The NNL solution was sterilised by filtration prior plating out by using 0.2 µm pore size disposable filters, supplied by Whatman International Ltd., Maidstone, UK. The plates were incubated in anaerobic conditions at 37 °C for 72 hours. An anaerobic system was achieved by using BR 38 (Oxoid Ltd.)

For *Lb. acidophilus* MRS agar CM 361 (Oxoid Ltd.) was also used, but the plates were incubated under aerobic condition at 37°C for 72 hours.

- (b) *Bifidobacterium* spp. were enumerated under similar conditions as described above for the NNL solution, but Paramomycin Sulphate P-9297 (Sigma Ltd.) was added to inhibit the growth of *Str. thermophilus*, and hence the solution is referred to as NNLP.

*Lb. acidophilus* was enumerated by using MRSM which was prepared in house using basically the same components as MRS agar except that the glucose was replaced by maltose 291314H (BDH Chemicals Ltd.). The plates were incubated under aerobic conditions at 37 °C for 72 hours.

*Str. thermophilus* was enumerated by using M-17 agar CM 785 (Oxoid Ltd.), and the plates were incubated at 37 °C for 72 hours under aerobic conditions.

- (c) The yoghurt starter organisms were enumerated following the method described by IDF (1988b).

### 3.7 Identification of Starter Cultures

Isolated colonies of micro-organisms of each starter culture used (see section 3.6) was partially identified, by using carbohydrate utilisation, enzymatic activity, and light and confocal microscopy.

#### 3.7.1 Isolation method and media used

In general the isolation procedure performed for all the micro-flora could be described as follows:

- After enumerating the starter cultures, Gram's stain was performed to several individual colonies.
- A loop full of colony was streaked onto a plate containing a specific agar as described in section 3.6.1, and the plates were then incubated at 37 °C for 18-24 hours.
- After the incubation period, individual colonies were sub-cultured into a Universal bottle containing 10 ml of a specific broth media (see Table 3.2), and incubated anaerobically, micro-aerophilically or aerobically depending on the individual micro-organism tested. The incubation temperature was 37°C for 18 to 24 hours.
- The next step was to harvest the micro-organisms from the broth culture which was achieved by centrifugation technique. A centrifuge equipment BTL model (Baird & Tatlock Ltd.) was calibrated at 2500 rpm for 3 min. The supernatant

portion (broth) was replaced with sterile distilled water which was then used to identify the micro-organisms. The samples of micro-organisms were then ready to carry out the identification tests.

**Table 3.2** Type of agar and broth used for isolation of different starter cultures.

Microorganism	Agar	Broth	Growth condition
<i>Str. thermophilus</i>	M-17 CM 785 (Oxoid Ltd.)	M-17 CM 817 (Oxoid Ltd.)	Aerobic
<i>Lb. acidophilus</i> (Enumeration)	MRSM agar (Prepared in house)	-	Aerobic
<i>Lb. acidophilus</i> (Isolation)	MRS CM 361 (Oxoid Ltd.)	MRS CM 359 (Oxoid Ltd.)	Microaerophilic <sup>a</sup>
<i>Bifidobacterium</i> spp.	MRS CM 785 (Oxoid Ltd.) with cystein (0.05g 100g <sup>-1</sup> )	MRS CM 359 (Oxoid Ltd.) with cystein (0.05g 100g <sup>-1</sup> )	Anaerobic

<sup>a</sup> Microaerophilic systems T BBL Campy Pak 71034 from Becton Dickinson Ltd., Cockeysville, Maryland 21030, USA were used.

### 3.7.2 Metabolic reactions

Miniature reaction strips from bioMérieux, Hampshire, UK, were used to characterise each individual micro-organism in the starter cultures. A brief description of the principle of these test strips were:

- API-ZIM: this is a semiquantitative micro-method designed to identify 19 enzymatic activity (see Appendix I). This test was used in all of the micro-organisms involved in the present study.
- Rapid ID-32A: these strips consist of 29 test cupules that contain de-hydrated test substrates, and the reading can be performed either using the ATB instruments or visually. Identification is then obtained referring to Analytical Profile Index (see Appendix II). These strips were used to characterise bifidobacteria species.



- API 50CH; this strip allows the study of carbohydrate metabolism of micro-organism. It consists of 50 micro-tubes each containing an anaerobic zone (the tube portion), for the study of fermentation and an aerobic zone (the cupule portion), for the study of oxidation and assimilation (see Appendix III). These strips were used to determine the carbohydrate metabolism in lactobacilli and bifidobacteria species.
- ID32 STREP; this is a system for identifying streptococci and related organisms in 4 hours. It consists of standardised and miniaturised enzymatic tests (see Appendix IV). This system was used for identifying streptococci in the present study.

In all cases the procedure followed the manufacturer's instructions, except in the rapid ID 32 STREP that M-17 Agar was used instead of Columbia Sheep Blood Agar because poor growth of the micro-organism was observed on Blood Agar, and in some instances no growth was observed.

### **3.7.3 Light microscopy**

A compound light microscope, Olympus Vanox (Olympus Optical Co., London, UK) was used to identify the morphological characteristics during the different stages of the isolation procedure of the micro-organisms. Gram's stains slides were prepared following the methodology described by Harrigan and McCance (1976). Heat-fixed smears from fresh cultures were prepared, and stained with Crystal violet Prod.35083 (BDH Chemical Ltd.) solution (0.5 w/v) for 1-2 min. After rinsing with water, Gram's Iodine 35015 4X (BDH Chemicals Ltd.) solution was added and left for 1 min. The iodine was then poured off and the slide was washed with alcohol and rinsed with tap water. Finally, the slides were stained with dilute Carbol Fuchsin 35008 (BDH Chemical Ltd.) solution for ~20 s, and then dried off prior microscopic examination.

### **3.7.4 Confocal microscopy**

A Laser Scanning Confocal Microscope (LSCM) MRC-1000 (BIO-RAD Microscience Ltd., Hemel Hemstea, Herts, UK) was used to identify the

morphological characteristics and determine approximate size of the micro-organisms. The slides were prepared by using the heat-fixed smear procedure described by Harrigan and McCance (1976) just before carrying out the metabolic tests (section 3.7.2). The samples were then stained by using Acridine Orange (1g per 1000g) solution and Glycerol AnalaR (BDH Chemicals Ltd.) at a rate of one part each. The images were recorded and stored on a hard disk of the computing system connected to the microscope.

### **3.8 Analysis of Milk Base**

#### **3.8.1 Chemical analysis**

Fat, total nitrogen, total solids and ash contents were determined according to the methods described in sections 3.4.1, 3.4.2, 3.4.3 and 3.4.4, respectively.

#### **3.8.2 Determination of the pH**

The pH values of each individual milk base just prior inoculation was performed as described in section 3.2.5.

#### **3.8.3 Determination of organic acids**

The organic acids content in the milk bases was determined according to the chromatographic method described by Marsili *et al.* (1981). However, the operation temperature in the column was modified to 72 °C, in order to get a better resolution of the peaks for orotic and citric acids.

A Spectra-Physics HPLC System (San Jose) was used, which consisted of an auto sampler (Model SP 8780 XR), a detector (model LC 871 UV-VIS-PYE Unicam Ltd.) and a pump (model SP 8770 isocratic- Santa Carla, California, USA). The column effluent was monitored at a wave length of 220 nm. Calculation of the organic acids was carried out by using a CHROM Perfect data processing system (Chrompack

International B.V., The Netherlands). Analyses were performed isocratically at a flow rate of 0.7 ml min<sup>-1</sup> and a temperature of 72 °C (in the column). The running time of each analysis was approximately 25 min.

The HPLC column used was a Chrompack (257016 Cat. No 28350), provided with polymeric packing specially designed for organic acids analysis. The length of the column was 300 mm, and the internal diameter 6.5 mm.

A calibration standard of aqueous organic acids solution containing the compounds of interest was made up. Such standard was made by using different concentrations of high purity components (analytical grade) of orotic, citric, pyruvic, lactic, uric, acetic, propionic, butyric and hippuric acids at the following concentrations: 10, 900, 57.7, 1200, 3, 842, 935.8, 1189 and 8.7 µg g<sup>-1</sup> respectively.

The procedure of sample preparation was as follows: 5g of milk base, 5 ml of distilled water, and 20 ml of acetonitrile HPLC grade (BDH Chemical Ltd.) were added to a 50 ml glass cylindrical tube. The mixture was rigorously shaken and then filtered through a 0.45 µm filter paper Wathman No 1 (Whatman International Ltd., Maidstone, UK). A 10 µL aliquot of filtrate was injected into the HPLC system for analysis.

#### 3.8.4 Enumeration of the starter cultures in the milk base

Enumeration of the starter cultures micro-flora (*i.e.* *Lb. acidophilus*, *Bifidobacterium* spp., *Str. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus*) in the milk base was carried out using different selective media and the pour-plate methods described in section 3.6.1. The counts were expressed as cfu g<sup>-1</sup>.

### **3.9 Analysis of Fresh and Stored Fermented Milk**

#### **3.9.1 Chemical analysis**

Fat, total nitrogen, total solids and ash contents of the fermented milks (fresh and stored) were determined, according to the methods described in sections 3.4.1, 3.4.2, 3.4.3 and 3.4.4, respectively. Organic acids and the pH values were determined according to the methods described in sections 3.8.3 and 3.2.5, respectively.

##### **3.9.1.1 Determination of whey proteins denaturation**

The degree of whey protein denaturation [*i.e.* Immunoglobulins (Ig),  $\alpha$ -lactoalbumin ( $\alpha$ -La), serum albumin lactoferrin (Sa/Lf) and  $\beta$ -lactoglobulin ( $\beta$ -La)] was determined according to the method described by Law *et al.* (1993) using the fast protein liquid chromatography (FPLC). The results were expressed as a percentage.

#### **3.9.2 Microbiological analysis**

Total viable count of non-lactic acid bacteria in the fermented milks (fresh and stored) were determined according to the IDF (1991b), and the growth medium used was plate count agar CM 325 (Oxoid Ltd.). Enterobacteria, yeasts and moulds counts were determined according to the methods described in sections 3.4.12 and 3.4.13, respectively.

#### **3.9.3 Enumeration of the starter cultures**

The enumeration of the starter micro-organisms in the fermented milks (fresh and stored) were performed following the method described in section 3.6. The media preparation and the incubation conditions were carried out according to the methods described in section 3.6.1. Gram's stain test (section 3.7.3) was performed to confirm the identity of each type of micro-organisms.

### **3.9.4 Syneresis (serum separation assessment)**

Serum separation, from the fermented milks (fresh and stored) was estimated by the method of Kessler (1981). The approximate weight of a hemisphere of product was 21 g, and the test was performed over a 2 h period at 5 °C. A stainless steel mesh size of 0.40 mm was used. The tests were performed after storage for 2, 5, 8, 13 and 20 days. Figure 3.1 (A) illustrates the equipment used for this analysis.

### **3.9.5 Firmness (compression response)**

A Stevens Texture Analyser (C. Stevens & Son Ltd., Hertfordshire, UK) was used to evaluate the firmness of the fermented milks when fresh and stored. The analyser was provided with a cylindrical probe (type TA3-TFE-105-504), 25 mm in diameter and 35 mm long. The probe was calibrated to penetrate the sample at a depth of 15 mm, speed 0.5 mm s<sup>-1</sup>, and the force exerted on the probe was recorded. An illustration of the analyser is shown in Figure 3.1 (B).

Firmness was evaluated on the samples just after removing them from the cold store at ~5 °C. The measurements were performed 2, 5, 8, 13 and 20 days after production, and the results were expressed in Newton (N).

### **3.9.6 Organoleptic assessment**

#### **3.9.6.1 Samples of fermented milks**

Fermented milks were stored at < 6°C in their containers until tested. Immediately before sensory assessment each container was labelled with a unique, encrypted code mark. Three separate experiments were carried out, some months apart, and in each trial the yogurt was evaluated (twice) after refrigerated storage for approximately 3, 10 and 17 days after manufacture. This testing schedule, involving 6 assessments for each

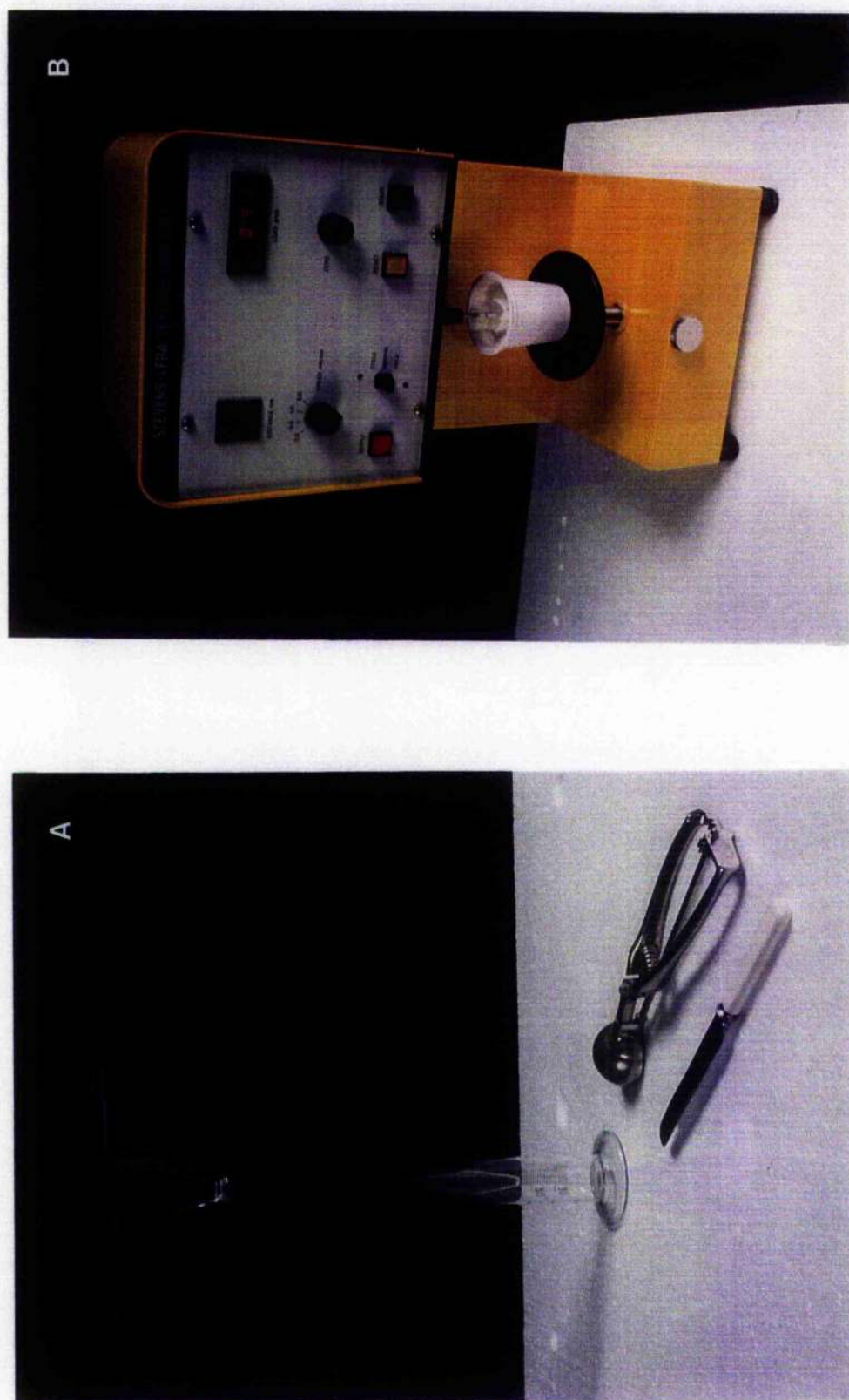


Figure 3.1 Illustrations of the equipment used for measuring syneresis (A) and firmness (B) of fermented milks.

trial, was designed to provide a balanced view of the product throughout its anticipated shelf life.

### 3.9.6.2 Sensory analysis

The development and application of an experimental vocabulary for profiling 'natural' fermented milks has been previously described by Muir and Hunter (1992). However, this vocabulary, comprising 32 attributes, was subsequently found to contain many redundant terms (Hunter and Muir, 1993). As a result, it was shown that the essential character of the natural product was described by a list of only 11 attributes. The vocabulary comprised:

- a. 3 attributes for aroma (*acid*, *creamy* and *sweet*)
- b. 4 flavour descriptors (*acid*, *creamy*, *sweet* and '*other*')
- c. 2 terms describing after-taste (*acid* and *natural*)
- d. 2 attributes encompassing physical properties (*viscosity* and *serum separation*)

Products were rated by a panel drawn from 16 female, external assessors highly-experienced and trained in profiling a wide range of foods and beverages. Because the trials were carried out over an extended period, the composition of the panel changed between trials (Table 3.3). Sensory profiling was carried out using a Design, Data capture and Analysis of Sensory Profiles protocol (DDASP) developed by Williams *et al.* (1996). In DDASP, experimental design, tasting instructions, sample rating, data collection and analysis are by means of an integrated computer-assisted system. Presentation order was balanced according to Williams (1949), MacFie *et al.* (1989) and Muir & Hunter (1991/2), to allow estimation of assessor, sample and order of tasting effects. Assessors were instructed to cleanse their palate with plain biscuit and cold, filtered tap water before profiling each sample. Each attribute within the vocabulary was rated on an undifferentiated 15 cm scale with anchor points.

**Table 3.3** Assessors used for product evaluation.

<b>Trial 1</b>	<b>Trial 2</b>	<b>Trial 3</b>
*	*	50
52	52	52
53	53	53
55	55	55
56	56	56
59	59	59
*	62	62
63	63	63
64	64	64
65	65	65
66	66	66
*	*	67
68	68	*
*	*	69
70	70	*
71	71	*
<b>Total = 12</b>	<b>Total = 13</b>	<b>Total = 13</b>



Four samples were profiled in each session and the experiment was replicated *i.e.* there were 4 sessions in each of 3 weeks. In total, 24 samples, comprising fermented milk made from 8 starter culture types were profiled.

Product characterisation was carried out under 'daylight' illumination in isolated, air-conditioned booths within a sensory laboratory.

### **3.9.7 Statistical analysis**

#### **3.9.7.1 Sensory evaluation**

Sample x replicate effects (144) were computed by the Residual Maximum Likelihood Technique (REML) as implemented in the Genstat suite of statistical software (Release 5.1; Copyright 1993, Lawes Agricultural Trust, Rothamsted Experimental Station). REML is a computationally-intensive analysis of variance (ANOVA) technique, particularly well suited to analysis of data which is not fully balanced (Patterson and Thompson, 1971; Horgan and Hunter, 1992). A mixed model was fitted: Assessor and assessor by replicate were designated random effects; Order of tasting, replicate, sample and sample by replicate were designated fixed effects. Mean culture x trial effects (24) were then computed and these data formed the basis of all further analyses (Appendix V).

First, the differences in individual attribute ratings between culture types were estimated using the General Linear Model (Minitab 9.2; Minitab Inc., State College, PA, USA) and the significance of the differences determined by a variance ratio test (F-test). Second, to aid interpretation of the data, a Principal Component Analysis (PCA; using the covariance matrix; Minitab 9.2) was executed using the culture effects (24) and further simplified by Factor Rotation (varimax). The significance of the individual Factors was judged by the amount of variance explained and by an analysis of variance of the culture effects. Interpretation of the individual Factors was made by inspection of the correlation of the object scores with the original attribute ratings.

### **3.9.7.2 Compositional quality**

The data of the compositional quality were analysed by univariate (analysis of variance, regression) and multivariate [Principal Component Analysis (PCA)] using the Genstat 5 Release 3.2 computer programme (copyright Lawes, 1990) Agricultural Trust, Rothamsted Experimental Station, and Minitab Release 10 (Minitab Inc., Pennsylvania State College, PA 16801, U.S.A).

## **CHAPTER FOUR:**

# **ENUMERATION AND IDENTIFICATION OF STARTER CULTURES**

## CHAPTER FOUR: ENUMERATION AND IDENTIFICATION OF STARTER CULTURES

### 4.1 Enumeration of Starter Cultures

As mentioned elsewhere, one of the aims of this project was to evaluate the number of viable lactic acid bacteria in the fermented milks. The media for the enumeration of *Lb. acidophilus*, *Str. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus* are well established, and more attention was paid to the media used for enumerating bifidobacteria species. Based on the reviews by Arroyo *et al.* (1994) and Rasic (1990) on the growth media available to enumerate *Bifidobacterium* spp. in fermented milks and taking into consideration the starter cultures supplier's recommendations, it was decided to use the media described in section 3.6.1. The enumeration procedure was carefully carried out under aseptic conditions following the methods described in sections 3.6 and 3.6.1.

To confirm the beneficial effect of adding cysteine to the media used (MRS, Oxoid) to grow *Bifidobacterium* species, enumeration tests were carried out in parallel by using media with the addition of cysteine (0.05g 100g<sup>-1</sup>) as a pure compound, L-hydrochloride cysteine or free of cysteine. The former two media have yielded larger size colonies ~2 mm in diameter, when compared with the media with no added cysteine. Such observation was for starters cultures DV B-100, MSK 2, and ABT-3. The size of colonies in the starter culture MSK B2 was tiny, but larger size was observed (~1 mm) when cysteine was present.

Table 4.1 shows the results of the counts of each starter culture used, including the approximate counts provided by the manufacturers. Slight variations were observed between the experimental counting and the starter cultures supplier's specifications. Although, slightly higher counts were found in the media containing cysteine, there was one exception where starter culture MSK 2 had no growth of *Bifidobacterium* spp. in the media which was not enriched with cysteine.

**Table 4.1** Enumeration of the lactic acid bacteria in the starter cultures including the suppliers' specifications (cfu g<sup>-1</sup>)<sup>f</sup>.

Code	<i>Bifidobacterium</i> spp.			<i>Lb. acidophilus</i>	<i>Str. thermophilus</i>	<i>Lb. delbrueckii</i> <i>ssp. bulgaricus</i>	Suppliers' specifications <sup>d</sup>
	a	b	c				
AB	3.6x10 <sup>11</sup>	4.7x10 <sup>11</sup>	5.4x10 <sup>11</sup>	1.4x10 <sup>11</sup>			5-20x10 <sup>10</sup>
AC/BL	9.2x10 <sup>8</sup>	1.1x10 <sup>9</sup>	1.1x10 <sup>9</sup>	5.6x10 <sup>9</sup>			~2x10 <sup>10</sup>
DV B-100	2.9x10 <sup>9</sup>	4.6x10 <sup>9</sup>	4.9x10 <sup>9</sup>	7.1x10 <sup>9</sup>	7.5x10 <sup>9</sup>		~1x10 <sup>10</sup>
MSK 2	NG <sup>e</sup>	4.3x10 <sup>7</sup>	4.8x10 <sup>7</sup>	6.9x10 <sup>9</sup>	4.3x10 <sup>7</sup>		1-5x10 <sup>8</sup>
ABT-1	3.4x10 <sup>9</sup>	3.4x10 <sup>9</sup>	4.1x10 <sup>9</sup>	1.2x10 <sup>10</sup>	4.2x10 <sup>10</sup>		5-20x10 <sup>10</sup>
ABT-3	4.5x10 <sup>9</sup>	5.7x10 <sup>9</sup>	6.0x10 <sup>9</sup>	2.1x10 <sup>10</sup>	1.7x10 <sup>11</sup>		5-20x10 <sup>10</sup>
MSK B2	3.2x10 <sup>8</sup>	3.6x10 <sup>8</sup>	3.7x10 <sup>8</sup>	4.8x10 <sup>8</sup>	8.7x10 <sup>7</sup>		1-3x10 <sup>8</sup>
MY 087					6.6x10 <sup>10</sup>	2.5x10 <sup>8</sup>	-

a Represent counts using media without cysteine.

b Represent counts using cysteine 0.05g 100g<sup>-1</sup>.

c Represent counts using L-cysteine hydrochloride 0.05g 100g<sup>-1</sup>.

f Results are average of two determinations performed on the same sample.

d Approximated counts for any micro-organism in the starter culture.

e No growth was observed at 10<sup>5</sup> dilution.

- No data available.

## 4.2 Identification of Starter Cultures

Isolates of micro-organisms from each starter culture were tested for morphological examinations, enzymatic activities, and carbohydrate utilisation, and the results were:

### 4.2.1 Microscopic analysis

Microscopy is widely used as a useful bacteriological technique to study the morphology of micro-organisms. Two techniques were used: (a) light microscopy (LM) and (b) confocal microscopy. As anticipated, all the isolates from the different starter cultures were Gram +ve.

The LM is a simple technique which allows to visualise the morphology of the cells of micro-organisms by examining a stained smear. This examination is often carried out at magnifications as high as 1000x when using the oil-immersion objective. There is a considerable literature of light microscopy observations of food and related materials (White and Shenton, 1976, 1982); however, it has certain limitations. For example, it lacks the resolution, and as a consequence, the isolates of bifidobacteria, streptococci and *Lb. delbrueckii* ssp. *bulgaricus* in some starter cultures (e.g. AB, ABT-1, ABT-3, MSK B2 and MY 087) could not differentiate accurately between strains and/or species. Thus, the isolates represent one strain only, and to map out each starter properly is time consuming and far beyond the scope of the present study. Therefore, the results shown in the subsequent sections may possibly identify in particular a specie present in the starter cultures containing bifidobacteria (see Table 3.1).

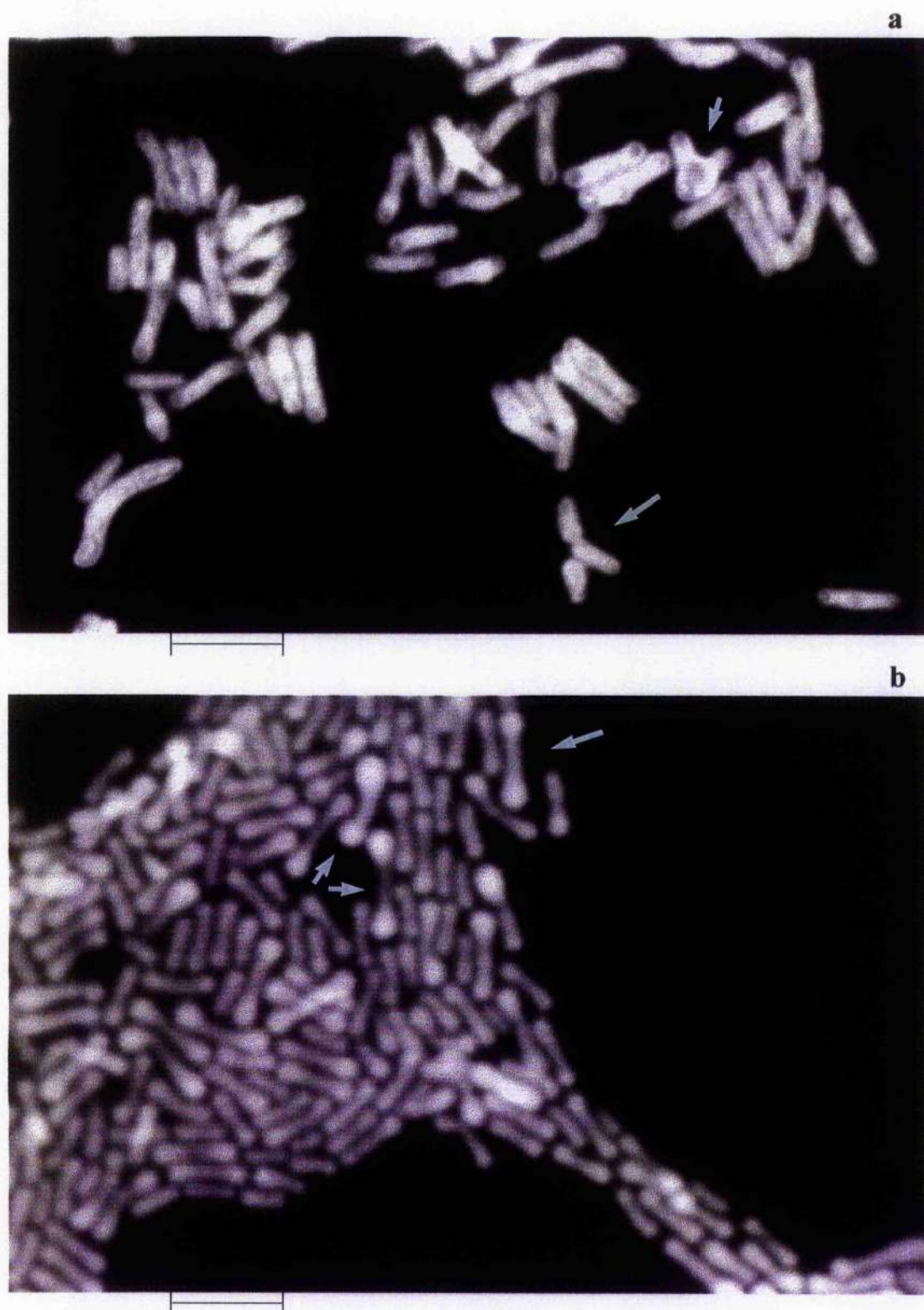
Light microscopy can be used by using different optical techniques such as bright-field, dark field, phase contrast, fluorescence and confocal microscopy. The latter is a recent development which improves the resolution of the classical light microscopy, and allows 3-D reconstruction. One of the main advantages of using this technique is that the images are measured in the form of electronic signal which allows a range of electronic image-processing techniques (Sheppard, 1993).

Electron microscopy allows to examine objects smaller than 0.2  $\mu\text{m}$  such as viruses and internal structures of cells. It uses a beam of electrons instead of light. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) offer higher resolution and usually require special sample preparation techniques. The former (SEM) allows to examine only surfaces, but the specimen preparation is relatively easier than in the latter microscopic method. Many applications of TEM to dairy products have been reported in reviews by Kalab (1993) and Brooker (1979). However, such microscopy techniques could not be used due to lack of facilities.

For confocal microscopy studies, fixed-smears were prepared by using the procedure described by Harrigan and McCance (1976). The slides were then stained with acridine orange and glycerol for 4-5 min (see section 3.7.4), and then observed under the microscope using an oil immersion objective. The images were then monitored by using the computing software CoMOS which allowed to improve the resolution and sharpness. The images were recorded on the hardware of the system and subsequently printed.

Figure 4.1 illustrates the morphology of *Bifidobacterium* species, and it can be observed that most of the strains had homogenous shape and size. For example, Figure 4.1 (a) where starter culture AB shows the typical shape of *Bifidobacterium* cells which are slightly swollen at either end of the cells, slim in the middle and in some cases forming "V" shapes (see arrows). They present variable sizes from  $\sim 2.5$  to 5  $\mu\text{m}$ . The cells in Figure 4.1 (b) of starter culture AC/BL shows a bone shape structure, very swollen at the extremities of the cell (see arrows), and the sizes ranged between 2.5 and 5  $\mu\text{m}$ ; however in some cases, "V" shapes can be observed. Similar morphology of the cells of the starter culture DV B-100 (Figure 4.1 c) was observed but in addition some cells show swollen structure in the middle (see arrows).

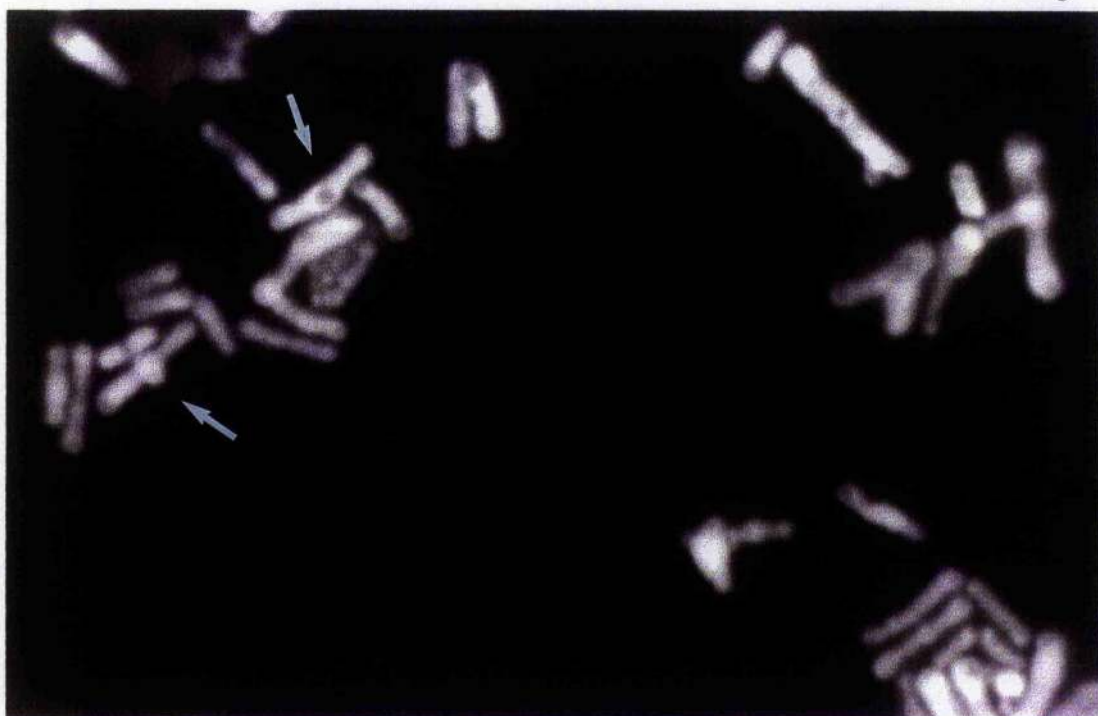
The morphology of the cells in Figure 4.1 (e) and (f) of starter cultures ABT-1 and ABT-3, respectively, are quite similar to each other presenting more or less the same characteristics of bifidobacteria as previously described. These *Bifidobacterium* species could be the same since both cultures were obtained from the same supplier.



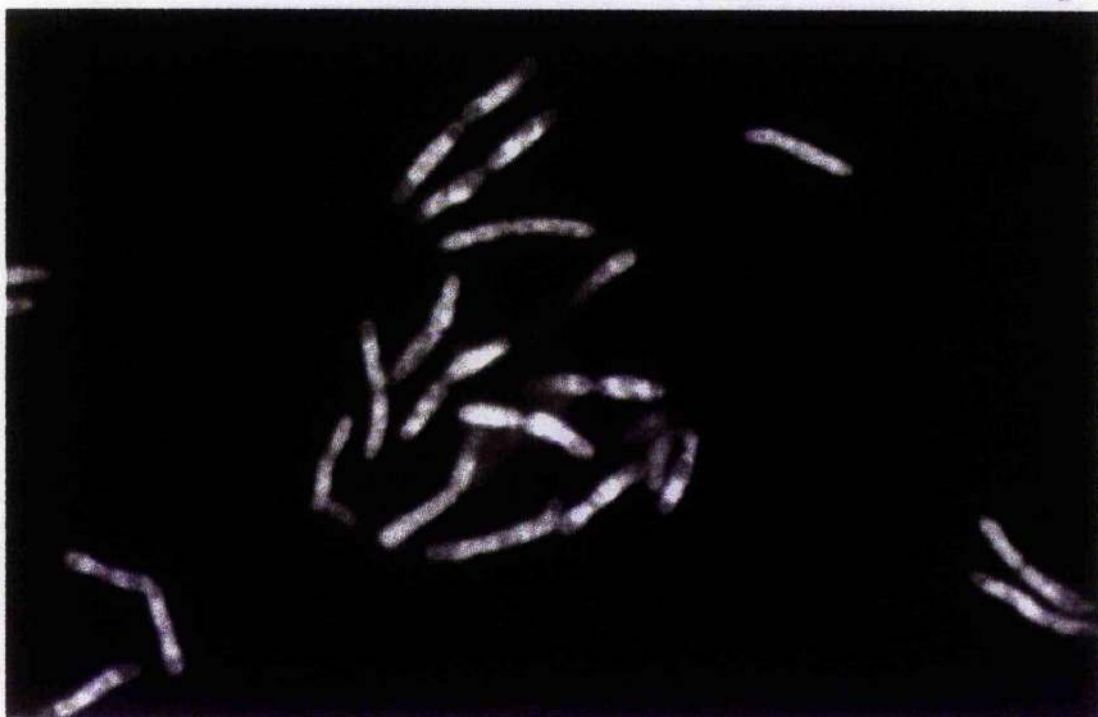
**Figure 4.1** The morphology of *Bifidobacterium* cells isolates from commercial starters cultures [(a) AB and (b) AC/BL], after subculturing twice using MRS medium supplemented with cysteine ( $0.05\text{g } 100\text{g}^{-1}$ ). Bar size =  $5\text{ }\mu\text{m}$ .



c

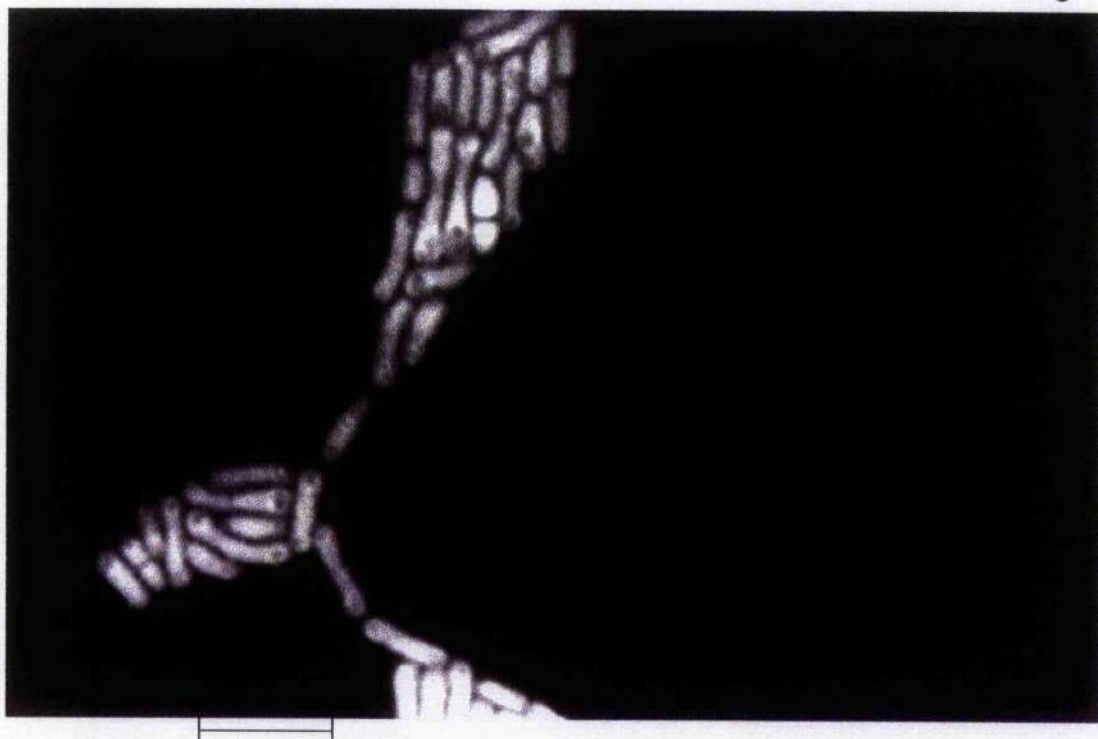


d



**Figure 4.1** (continued) [(c) DV B-100 and (d) MSK 2].

e

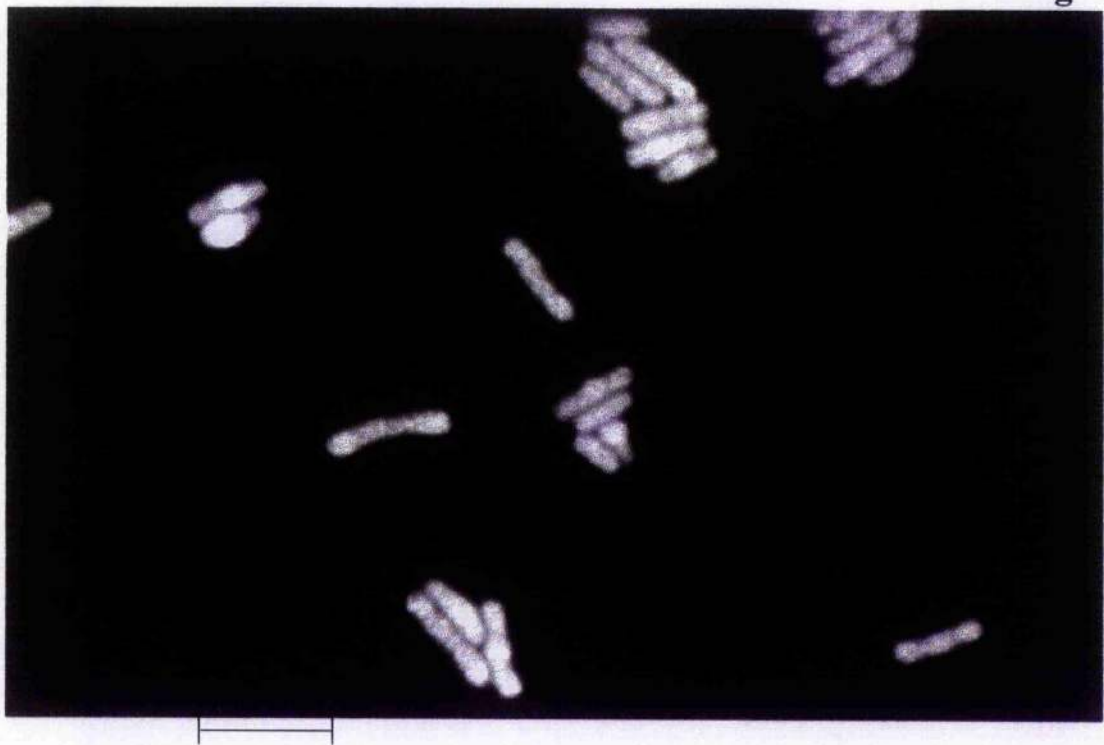


f



Figure 4.1 (continued) [(e) ABT-1 and (f) ABT-3].

g



**Figure 4.1** (continued) [(g) MSK B2].

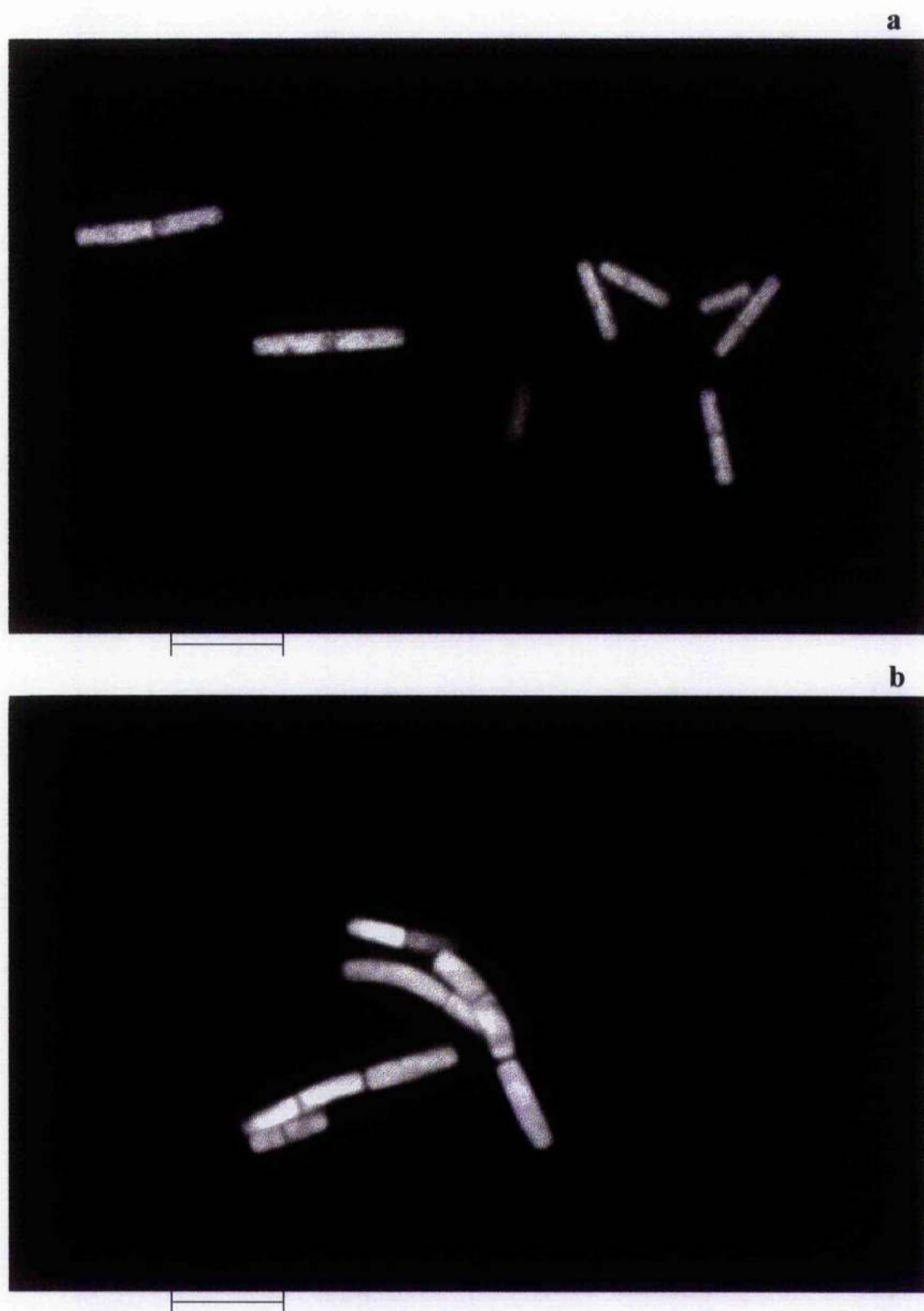
Similar morphological pattern could also be observed for isolates from starter culture MSK B2 (Figure 4.1 g), but they were slightly smaller in size ( $\sim 1.5\text{--}4\text{ }\mu\text{m}$ ). It is more likely that swollen cells of bifidobacteria isolates from starter cultures AB, ABT-1, ABT-3 and MSK B2 could be *Bifidobacterium animalis* (Sgorbati *et al.*, 1995), but other tests should be used in order to identify these isolates at species level. However, *Bifidobacterium* cells of the starter culture MSK 2 (Figure 4.1 d) had different morphology, almost straight rods, thin and filament “tails” like structures at their extremities which could be differentiated from the other isolates.

Figure 4.2 illustrates the morphology of *Lb. acidophilus* strains which look homogeneous with respect to their shape. Straight rods, in pairs, short chains or singles cells. Although the cells of starter cultures AC/BL and MSK 2 are slightly curved (Figure 4.2 b and d, respectively) and thicker than the rest, the sizes were variable from approximately  $2.5\text{ }\mu\text{m}$  (Figure 4.2 g) to  $6\text{ }\mu\text{m}$  in Figure 4.2 (e). Figure 4.3 illustrates the morphology of *Lb. delbrueckii* ssp. *bulgaricus* cells which had a typical large rod shape. The size was  $\sim 7\text{ }\mu\text{m} \times 2\text{ }\mu\text{m}$  which were differentiated very easily from the *Lb. acidophilus* cells.

Figure 4.4 illustrates the morphology of the *Str. thermophilus* cells which were very similar to each other. Typical coccus and ovoid shapes ( $\sim 1\text{--}2\text{ }\mu\text{m}$ ) forming chains and in some cases clusters. Some of the cells illustrate the cell division stage of reproduction (see arrows in Figures 4.4 b, c and f).

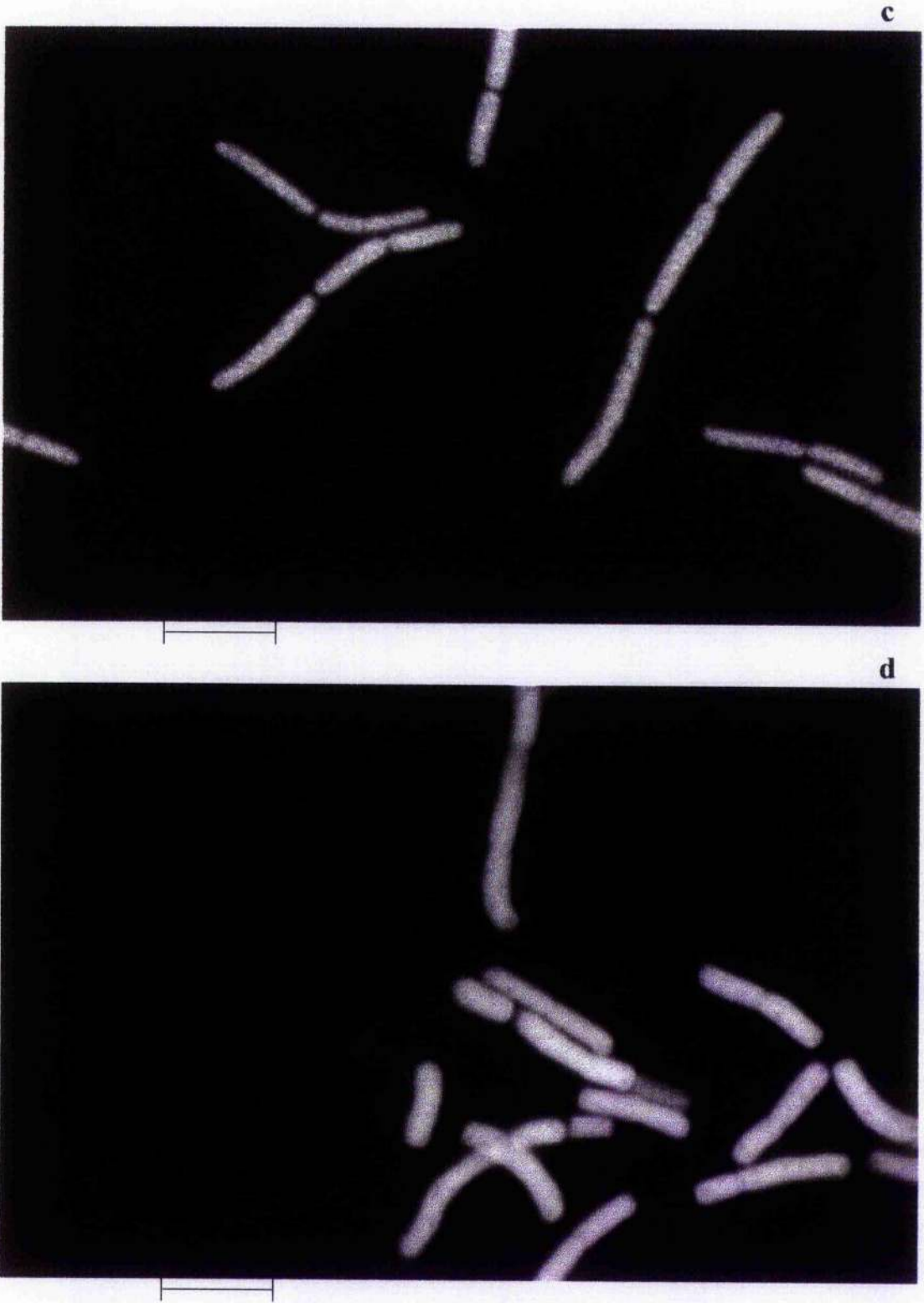
#### 4.2.2 Enzymatic activities

Different tests were performed on the isolates from the commercial starter cultures in order to profile their enzymatic activities, and if possible to use the results for identification purposes. These tests included the API-ZYM, ID 32A, ID 32 STREP and API 50 CHL. The isolation of the micro-organisms and the preparation of the inoculum were performed following the methods described in sections 3.7.1 and 3.7.2; however, details regarding the tests results are shown in Appendices I to IV. These enzymatic tests could be briefly described as follows:



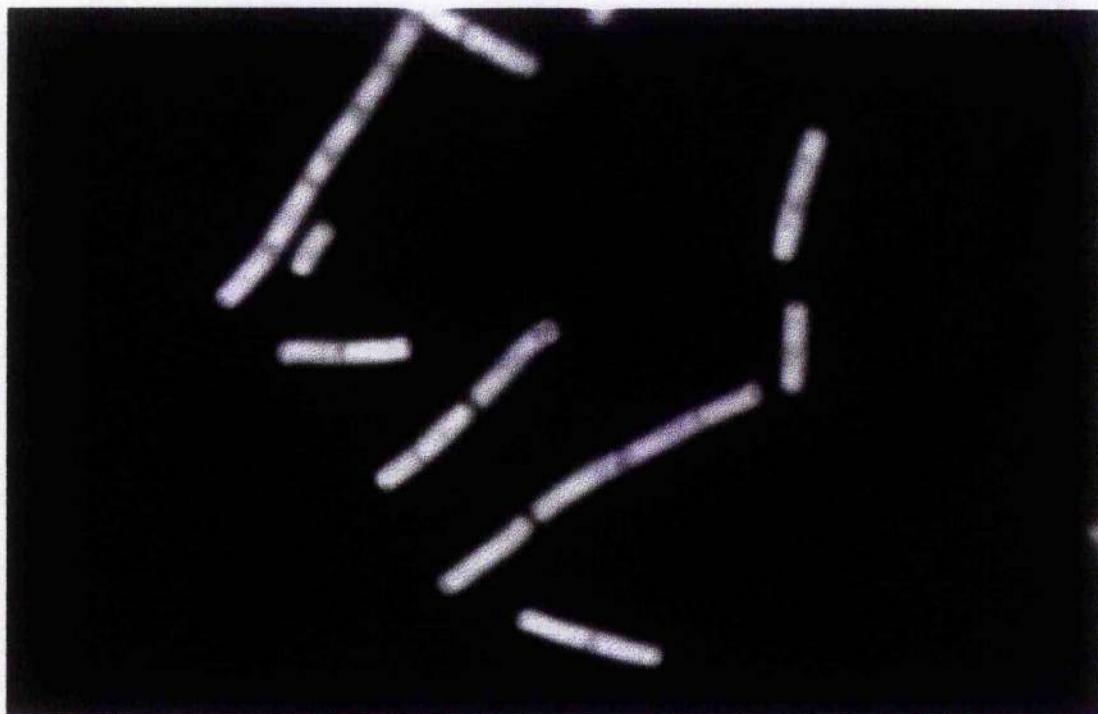
**Figure 4.2** The morphology of *Lb. acidophilus* cells isolates from commercial starter cultures [(a) AB and (b) AC/BL ] after subculturing twice using MRS medium. Bar size = 5μm.



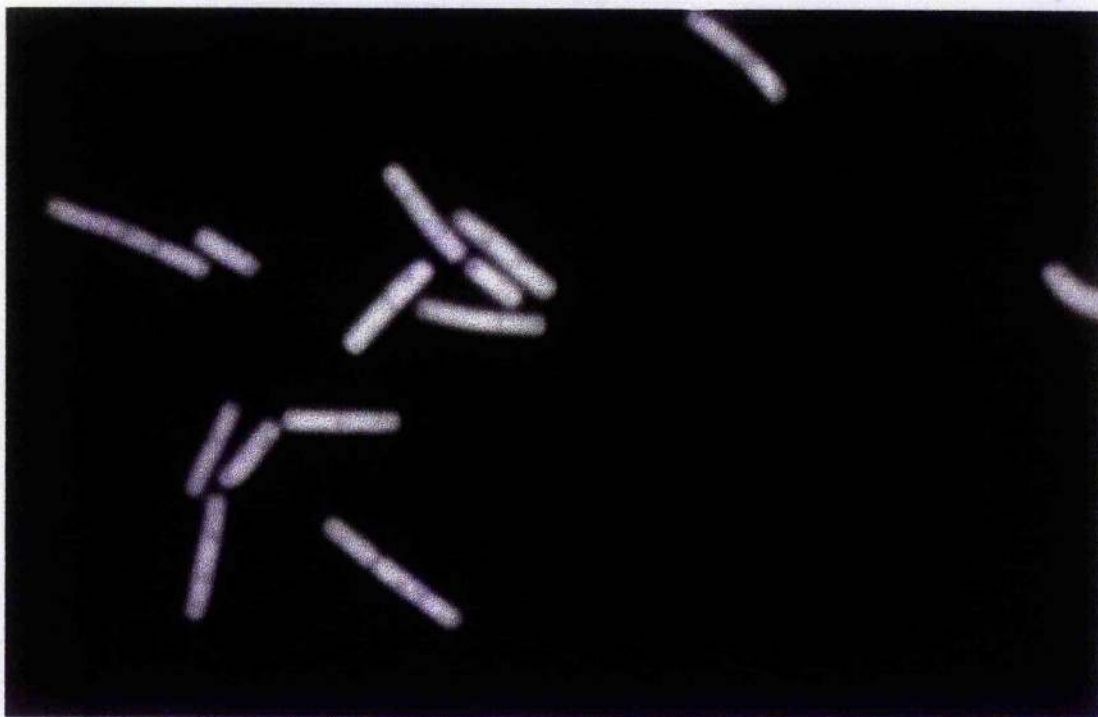


**Figure 4.2** (continued) [(c) DV B-100 and (d) MSK 2].

e



f



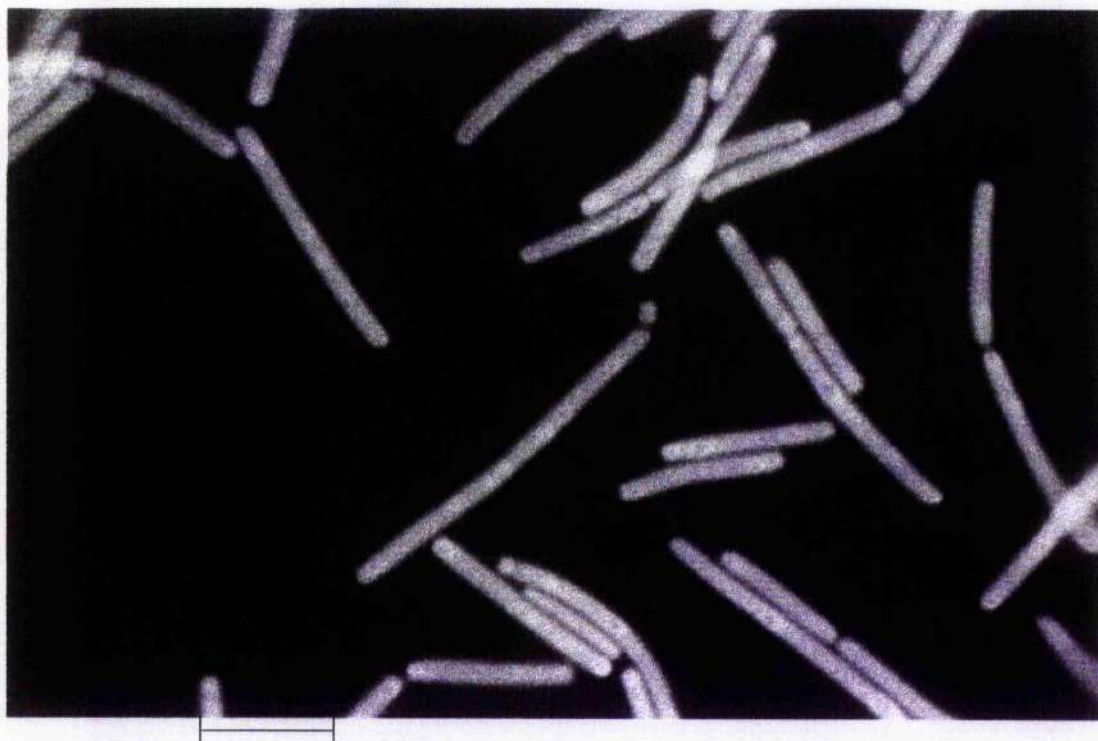
**Figure 4.2** (continued) [(e) ABT1 and (f) ABT3].

g

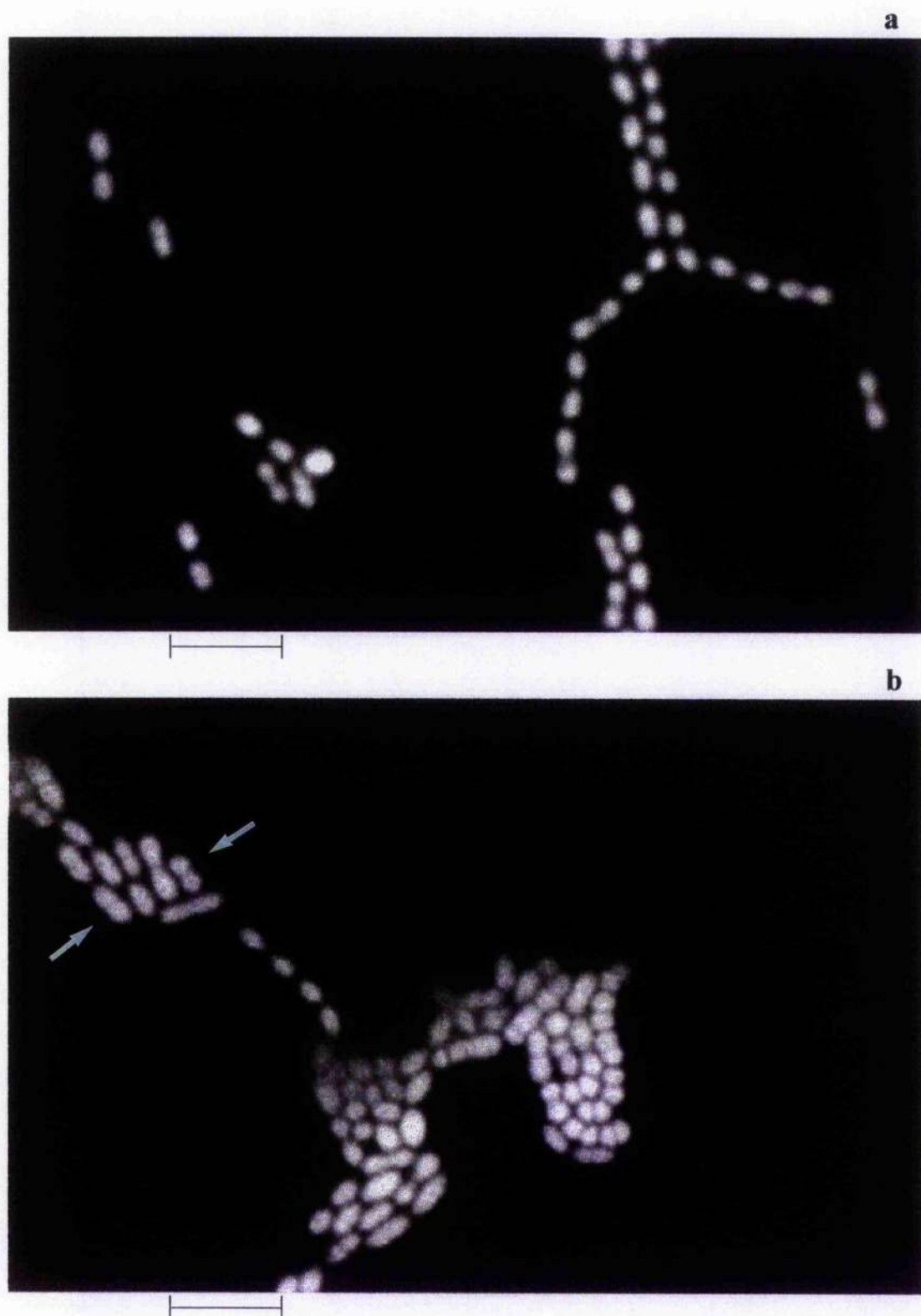


**Figure 4.2** (continued) [(g) MSKB2].

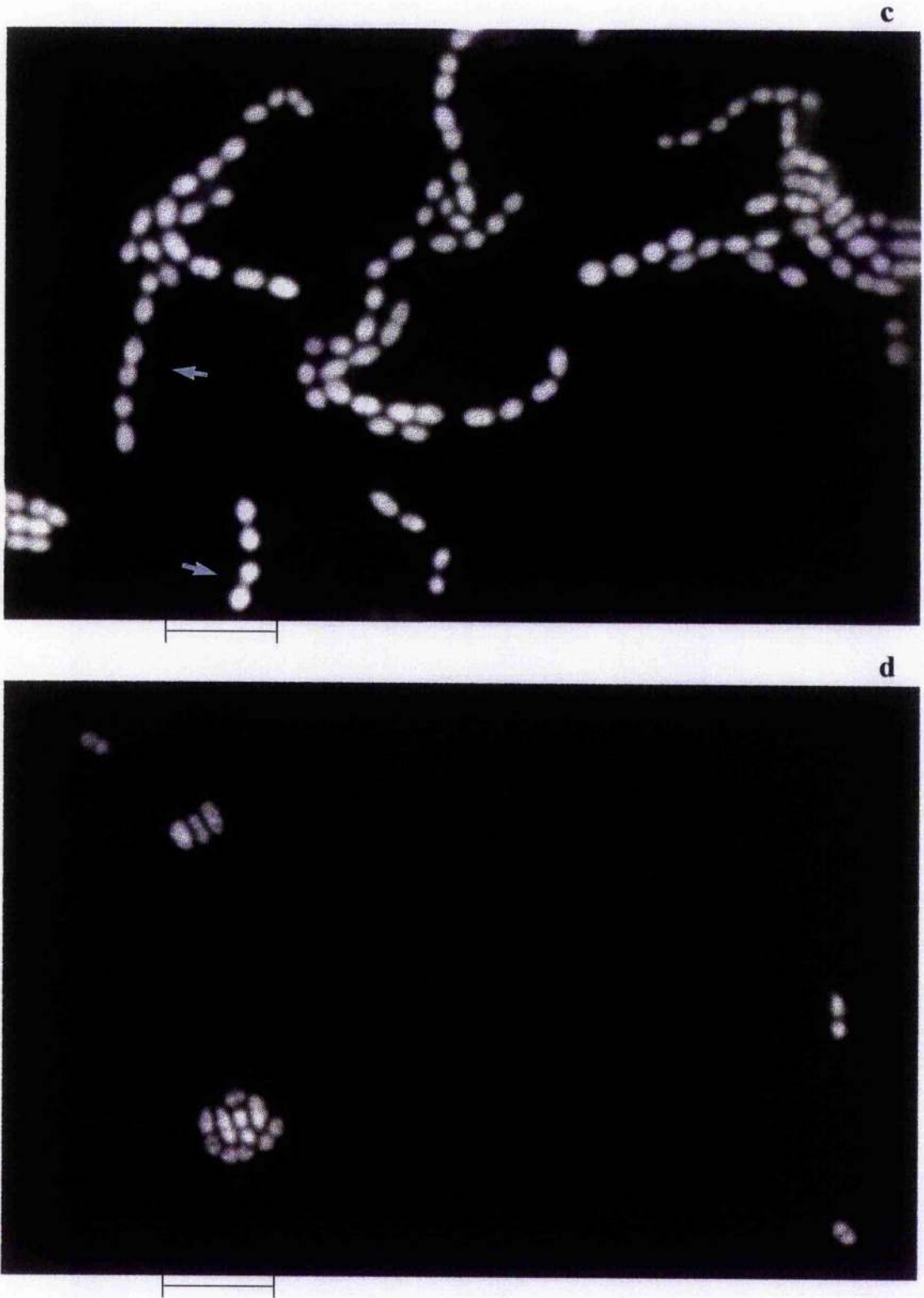




**Figure 4.3** The morphology of *Lb. delbrueckii* ssp. *bulgaricus* cells isolates from commercial starter culture (MY 087) after subculturing twice using MRS medium. Bar size 5 $\mu$ m.



**Figure 4.4** The morphology of *Str. thermophilus* cells isolates from commercial starter cultures [(a) DV B-100 and (b) MSK 2] after subculturing twice using M-17 medium. Bar size 5 μm.



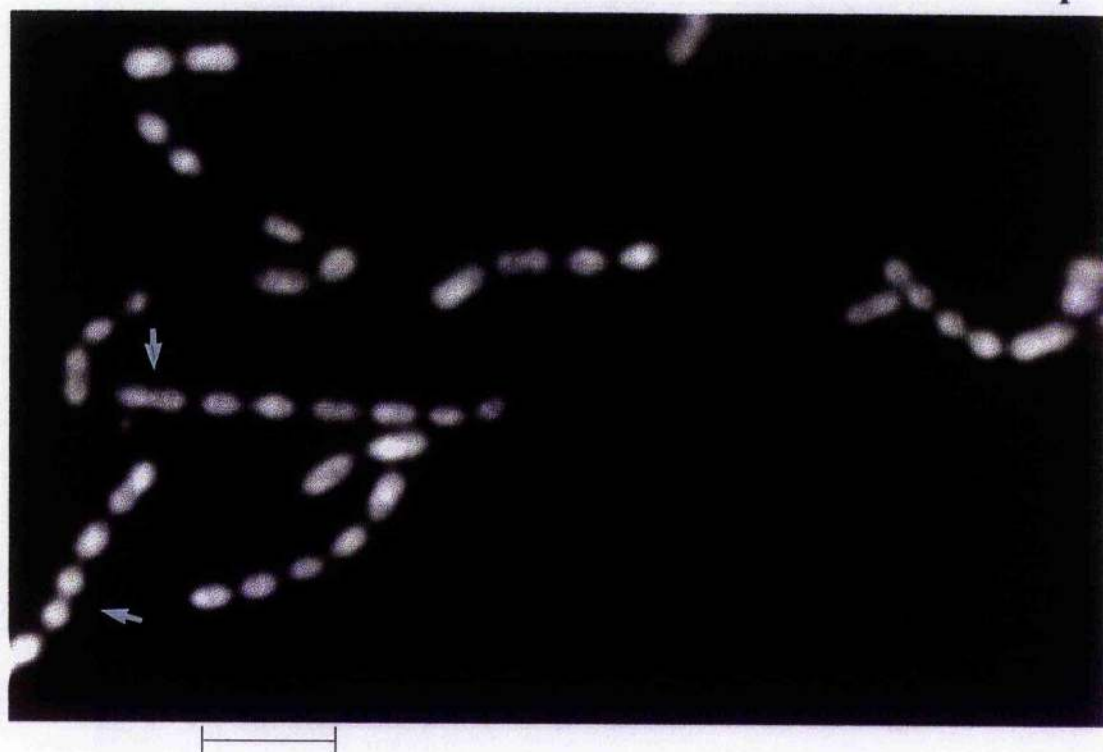
**Figure 4.4** (continued) [(c) ABT-1 and (d) ABT-3].



e



f



**Figure 4.4** (continued) [(e) MSK B2 and (f) MY 087].

*Firstly*, the API-ZYM test method is a semi-quantitative micro method which is used to determine some enzymatic activities that micro-organisms may possess. The API-ZYM strips were incubated for 4 h at 37 °C, and the enzymatic activity was expressed in a range of zero (negative reaction) and five (maximum reaction); however, readings of one through to four correspond to intermediate reactions.

*Secondly*, the ID 32A test method is designed for anaerobic micro-organisms which was used specially for the identification of bifidobacteria species beside the other tests used.

*Thirdly*, the ID 32 STREP test method was used specifically for the identification of *Streptococcaeae*, *i.e.* *Str. thermophilus*.

*Fourthly*, the API 50 CHL test method was used to evaluate the carbohydrate utilisation of the lactic acid bacteria isolates. The results were as follows:

#### 4.2.2.1 Enzymatic activities of bifidobacteria isolates

A wide range of enzymatic activities of *Bifidobacterium* species have been detected, and the results are shown in Table 4.2. However, similar results were reported by Bezkorovainy (1989) and Desjardins *et al.* (1990), but very weak activity for alkaline phosphatase was reported for *Bif. bifidum* and greater activity for N-acetyl- $\beta$ -glucosaminidase for both *Bif. infantis* and *Bif. bifidum* (see Table 4.2). Valine and cystine aminopeptidases activities (*i.e.* weak reaction) were reported for isolates from starter cultures AB and ABT-1, but not for the strains tested by Desjardins *et al.* (1990). This could be attributed to variation in enzymatic activities between strains of the same and/or different species (Roy *et al.*, 1992).

$\beta$ -galactosidase activity and to a lesser degree  $\alpha$ -glucosidase activity were very strong and consistent among the isolates tested (Table 4.2), and similar results were reported by Desjardins *et al.* (1990) and Chevalier *et al.* (1990). Leucine aminopeptidase activity (*i.e.* intermediate to maximum) was also detected in most of the starter cultures isolates except the strain from starter MSK 2. The same strain (MSK 2) showed higher  $\alpha$ -galactosidase activity when compared with the other

**Table 4.2** Characterisation of the enzymatic activity (API-ZYM) of different *Bifidobacterium* species<sup>a</sup>.

Enzyme	Commercial species of <i>Bifidobacterium</i>							ATCC <sup>b</sup> strains		
	AB <i>Bif. spp.</i>	ABT-1 <i>Bif. spp.</i>	ABT-3 <i>Bif. spp.</i>	MSK 2 <i>Bif. longum</i>	MSK B2 <i>Bif. spp.</i>	AC/BL <i>Bif. longum</i> <i>Bif. infantis</i>	DV B-100 <i>Bif. bifidum</i>	<i>Bif. longum</i> (two)	<i>Bif. infantis</i> (four)	<i>Bif. bifidum</i> (three)
Phosphatase alkaline	0	0	0	0	0	0	0	0	0	0↔1
Esterase (C <sub>4</sub> )	2	2	1	2	2	1	2	0↔1	1↔2	1↔2
Esterase lipase (C <sub>8</sub> )	1	1	1	2	1	1	1	2	1↔2	1↔2
Lipase (C <sub>14</sub> )	0	0	0	0	0	0	0	1↔2	0	0↔1
Leucine aminopeptidase	5	4	3	1	3	3	3	1	1↔3	1↔2
Valine aminopeptidase	1	1	0	0	0	0	0	0	0	0
Cystine aminopeptidase	1	1	0	0	0	0	0	0	0	0
Phosphatase acid	1	1	1	1	0	1	1	1	0↔2	1↔2
Naphthol-AS-Bi-phosphohydrolase	2	2	2	0	1	1	2	1	1↔2	1
α-galactosidase	2	2	2	3	2	1	2	3	2↔4	1↔3
β-galactosidase	5	5	5	4	5	5	5	4↔5	4↔5	5
α-glucosidase	3	4	3	4	2	2	2	4	3↔5	2↔4
β-glucosidase	2	2	2	0	1	1	1	0	0↔3	0
N-acetyl-β-glucosaminidase	0	0	0	0	0	0	0	0	1↔3	1↔5

<sup>a</sup> Numbers 1 to 4 indicate intermediate reactions (5-30 nM), number 5 indicates maximum activity (40 nM) and zero indicates a negative reaction.

<sup>b</sup> American Type Culture Collection; data compiled from Desjardins *et al.* (1990); the data between arrows indicate variation in enzymatic activity of different strains tested.

Results are approximate average of two determinations performed on the same sample.

No activity was found for trypsin, chymotrypsin, β-glucuronidase, α-mannosidase, or α-fucosidase.

isolates of bifidobacteria; however starter AC/BL showed the weakest reaction. With regard to the esterase C(4) and esterase lipase C(8) activities, all the bifidobacteria isolates showed an intermediate reaction, but the pattern of activity was variable among the isolates. Similar phenomenon was observed for  $\beta$ -glucosidase and naphtol-AS-BI-phosphohydrolase activities with the exception of starter culture MSK 2 which gave negative reaction.

An almost identical pattern of activity was observed for starter cultures AB, ABT-1, and ABT-3 suggesting that they are the same species of bifidobacteria. Incidentally, all these starter cultures were obtained from the same supplier.

The results obtained using the rapid ID 32A test method are shown in Table 4.3, and it can be observed that a consistent pattern exists in relation to  $\beta$ -galactosidase,  $\alpha$ -glucosidase, arginine aminopeptidase, leucine aminopeptidase and histidine aminopeptidase activities in most of the isolates from the different starter cultures. However, slight variations were observed in relation to  $\alpha$ -galactosidase and  $\beta$ -glucosidase activities where in the former enzyme activity the organisms from starter cultures ABT-1 and MSK B2 were negative, and in the latter enzyme activity negative reactions were also observed for starter cultures MSK 2 and ABT-1. Nevertheless, in the rest of the micro-organisms tested, positive reactions were observed for  $\alpha$ -galactosidase and  $\beta$ -glucosidase. Similar pattern was also observed during the fermentation of mannose and raffinose; isolates from starter cultures AB and ABT-3 strains gave positive reaction whilst the rest negative (*i.e.* for mannose fermentation), and only starter cultures AB, DV B-100 and ABT-1 gave negative reaction for raffinose fermentation. Such enzymatic activities may suggest that bifidobacteria isolates from starter AB & ABT-1 and ABT-3 & MSK B2 are closely related or similar (see Table 4.2).

It is evident that the results obtained using the API-ZYM test method (Table 4.2) was difficult to predict the exact nomenclature at species level. However, from the API-ZYM results it is safe to conclude that the isolates from starter cultures MSK 2,

**Table 4.3** Enzymatic activity of *Bifidobacterium* isolates using the rapid ID 32A system<sup>a</sup>.

	AB <i>Bif. spp.</i>	ABT-1 <i>Bif. spp.</i>	ABT-3 <i>Bif. spp.</i>	MSK 2 <i>Bif. longum</i>	MSK B2 <i>Bif. spp.</i>	AC/BL <i>Bif. longum</i> <i>Bif. infantis</i>	DV B-100 <i>Bif. bifidum</i>
Arginine di-hydrolase	-	-	-	-	-	-	~
$\alpha$ -Galactosidase	+	-	+	+	-	+	+
$\beta$ -Galactosidase	+	(+)	+	+	+	+	+
$\alpha$ -Glucosidase	+	(+)	+	+	+	+	+
$\beta$ -Glucosidase	+	-	+	-	+	+	(+)
$\alpha$ -Arabinosidase	+	+	+	+	+	+	+
Mannose fermentation	+	-	+	-	-	-	-
Raffinose fermentation	-	+	-	+	+	+	-
Arginine aminopeptidase	+	+	+	(+)	+	+	+
Proline aminopeptidase	+	~	+	+	+	+	+
Phenylalanine aminopeptidase	+	+	+	+	+	+	+
Leucine aminopeptidase	+	+	+	+	+	+	+
Tyrosine aminopeptidase	+	+	+	(+)	+	+	+
Glycine aminopeptidase	~	~	-	~	~	~	~
Histidine aminopeptidase	+	+	+	(+)	+	+	+
Serine aminopeptidase	+	+	+	~	+	+	+

+ Represents positive reaction.

(+) Represents strong but not complete reaction.

~ Represents weak reaction.

- Represents negative reaction.

<sup>a</sup> Results are approximate average of two determinations performed on the same sample.

No activity or reaction was observed for urease,  $\beta$ -galactosidase-6-phosphate, glutamic ac. decarboxylase,  $\alpha$ -fucosidase, reduction of nitrates, indole production, phosphatase alkaline, pyroglutamic ac. aminopeptidase, leucyl glycine aminopeptidase, alanine aminopeptidase, glutamyl glutamic ac. aminopeptidase,  $\beta$ -glucuronidase, or  $\beta$ -N-acetyl-glucosaminidase.



AC/BL and DV B-100 when compared with the data reported by Desjardings *et al.* (1990) are *Bif. longum*, *Bif. infantis* and *Bif. bifidum*, respectively. Consequently, the data reported in Table 4.3 was compared with a computer software (bio-Mérieux Ltd.), and the out-put had limited identification of bifidobacteria. The rapid ID 32A test method is capable of identifying bifidobacteria as *Bif. adolescentis* 1, *Bif. adolescentis* 2, or *Bifidobacterium* species, and none of the isolates belonged to *Bif. adolescentis*.

The results of the API 50 CHL test method for the utilisation of different carbohydrates by all the isolates of bifidobacteria is shown in Table 4.4. It can be observed that the isolate from starter culture MSK 2 had a very different pattern of carbohydrate hydrolysis when compared with the rest of the isolates. For, example such organism showed (a) negative reactions for amigdaline and esculine and (b) very weak reactions for D-turanose and L-xylose. Maltose, melibiose, D-raffinose, sucrose and D-glucose were metabolised by all the isolates, but the strains from the starter cultures AC/BL and MSK B2 showed a very weak reaction to D-glucose. Amigdaline was highly utilised only by the isolate from MSK B2 starter culture. There were many other sugars (*i.e.* L-arabinose, fructose, galactose, melizitose, ribose and D-xylose) that have been only metabolised by the isolate of MSK 2 starter culture, but not the other strains. Contrary to expectations, lactose has been only metabolised by the isolates from the starter cultures MSK 2 and ABT-1 (Table 4.4).

The results shown in Table 4.4 were compared with data reported in the literature (see Table 2.6), and the only isolates of bifidobacteria, which were identified as *Bif. longum*, *Bif. infantis* and *Bif. bifidum*, were from the starter cultures MSK 2, AC/BL and DV B-100, respectively, but not the rest.

#### 4.2.2.2 Enzymatic activities of lactobacilli isolates

The profiles of the enzymatic activities (API-ZYM) of *Lb. acidophilus* strains and *Lb. delbrueckii* ssp. *bulgaricus* are shown in Table 4.5. The leucine aminopeptidase activity in all of the *Lb. acidophilus* appeared to be very consistent

**Table 4.4** Carbohydrates metabolism (API 50 CHL) of *Bifidobacterium* species isolates from commercial starter cultures<sup>a</sup>.

	AB <i>Bif. spp.</i>	ABT-1 <i>Bif. spp.</i>	ABT-3 <i>Bif. spp.</i>	MSK 2 <i>Bif. longum</i>	MSK B2 <i>Bif. spp.</i>	AC/BL <i>Bif. longum</i> <i>Bif. infantis</i>	DV B-100 <i>Bif. bifidum</i>
L-arabinose	-	-	-	+	-	-	-
Amigdaline	~	~	~	-	+	~	~
Esculine	+	+	+	-	+	+	+
Fructose	-	-	-	+	-	-	-
Galactose	-	-	-	+	-	-	-
D-glucose	+	+	+	+	~	~	+
Lactose	-	+	-	+	-	-	-
Maltose	+	+	+	+	+	+	+
Melizitose	-	-	-	+	-	-	-
Melibiose	+	+	+	+	+	+	+
D-raffinose	+	+	+	+	+	+	+
Ribose	-	-	-	+	~	~	-
Sucrose	+	+	+	+	+	+	+
D-turanose	-	-	-	~	-	-	-
L-xylose	-	-	-	~	-	-	-

+ Denotes a positive reaction.

- Denotes a negative reaction.

~ Denotes a weak reaction.

<sup>a</sup> Results are approximate average of two determinations performed on the same sample.

No reaction was observed for cellobiose, gluconate, inulin, mannitol, mannose, salicine, sorbitol, trehalose, or D-xylose.

**Table 4.5** Profiles of enzymatic activities (API-ZYM) of *Lb. acidophilus* and *Lb. delbrueckii* ssp. *bulgaricus*<sup>a</sup>.

Enzyme	AB	ABT-1	ABT-3	MSK 2	MSK B2	AC/BL	DV B-100	MY 087 <sup>b</sup>
Esterase (C4)	1	1	1	1	1	1	1	1
Esterase lipase (C8)	1	1	0	1	0	1	1	0
Leucine aminopeptidase	3	3	3	3	2	2	2	1
Valine aminopeptidase	2	2	1	2	1	1	0	0
Cystine aminopeptidase	2	2	1	2	1	1	1	0
Phosphatase acid	2	2	1	2	1	1	1	1
Naphtol-AS-BI-phosphohydrolase	2	2	2	1	1	2	2	4
$\alpha$ -galactosidase	0	0	0	1	0	0	0	1
$\beta$ -galactosidase	1	1	2	1	3	1	2	0
$\alpha$ -glucosidase	1	1	1	1	2	1	1	2
$\beta$ -glucosidase	1	0	1	1	1	0	1	0
N-acetyl- $\beta$ -glucosaminidase	0	0	0	0	1	0	1	0

<sup>a</sup> Numbers 1 to 4 indicate intermediate reactions (5-30 nM), number 5 indicates maximum activity (40 nM) and zero indicates negative reaction.

<sup>b</sup> Corresponds to *Lb. delbrueckii* ssp. *bulgaricus* activity.

Results are approximate average of two determinations performed on the same sample. No activity was found for phosphatase alkaline, lipase (C<sub>14</sub>), trypsin, chymotrypsin,  $\beta$ -glucuronidase,  $\alpha$ -mannosidase, and  $\alpha$ -fucosidase.

showing a reaction strength ranging between 2 and 3. However isolates from starter cultures DV B-100, ABT-3 and MSK B2 have shown some degree of activity (*i.e.* between 2 and 3) of  $\beta$ -galactosidase, while the isolates from AB, AC/BL, MSK 2 and ABT-1 showed a very poor reactions. Valine aminopeptidase and cystine aminopeptidase have similar pattern of activity (*i.e.* between 1 and 2) in most of the strains which were isolated with the exception of the isolate from starter culture DV B-100 where no activity of valine aminopeptidase was detected (Table 4.5). The  $\alpha$ -glucosidase and  $\beta$ -glucosidase activities in most of the *Lb. acidophilus* isolates were also poor although the strain from starter MSK 2 showed slightly higher activity when compared with the rest. No activity was detected for  $\beta$ -glucosidase in the strains isolated from starter cultures AC/BL and MSK 2 (see Table 4.5).

Only one strain of *Lb. delbrueckii* ssp. *bulgaricus* (*i.e.* from starter culture MY 087) was tested, and showed a very strong activity for naphtol-AS-BI-phosphohydrolase. This isolate did not possess enzymatic activity for esterase (C8), valine aminopeptidase, cystine aminopeptidase,  $\beta$ -glucosidase, N-acetyl- $\beta$ -glucosidase and  $\beta$ -galactosidase (Table 4.5). However, with regard to the latter activity such observation was contrary to what have been reported in the literature (Tamime and Robinson, 1985) possibly due to the characteristic of such isolate or sensitivity of such test kit. Nevertheless, the same isolate showed a strong reaction for galactose, utilisation (see Table 4.6). Furthermore, intermediate reactions for esterase (C4), leucine aminopeptidase, phosphatase acid, and  $\alpha$ -galactosidase activities of the isolate *Lb. delbrueckii* ssp. *bulgaricus* from starter culture MY 087 were observed (see Table 4.5).

Table 4.6 illustrates the patterns of sugar metabolism of 7 strains of *Lb. acidophilus* and one *Lb. delbrueckii* ssp. *bulgaricus* using the API 50 CHL test method. The results of carbohydrate metabolism by *Lb. acidophilus* isolates were more consistent when compared with the results of bifidobacteria, but some variations were observed. For example, the isolate from starter culture MSK 2 was unable to metabolise sucrose and trehalose, but it was able to utilise D-mannose while the rest

**Table 4.6** Carbohydrates metabolism (API 50 CHL) by different isolates of *Lb. acidophilus* and *Lb. delbrueckii* ssp. *bulgaricus*<sup>a</sup>.

Carbohydrate	AB	ABT-1	ABT-3	MSK 2	MSKB 2	AC/BL	DV B-100	MY 087 <sup>b</sup>
Amigdaline	(+)	(+)	+	~	(+)	(+)	-	-
Cellobiose	+	(+)	+	(+)	+	+	+	-
Esculine	+	+	+	(+)	+	+	+	-
D-fructose	+	+	+	+	+	+	+	+
Galactose	(+)	+	+	+	+	+	(+)	(+)
$\beta$ -gentibiose	(+)	(+)	+	(+)	+	(+)	-	-
D-glucose	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	(+)	+
D-mannose	-	-	~	(+)	-	-	~	(+)
N-acetyl glucosamine	+	+	+	+	+	~	(+)	+
D-raffinose	+	+	+	+	+	(+)	(+)	+
Ribose	-	-	-	-	-	-	-	-
Salicine	+	+	+	~	+	+	-	+
Sucrose	+	+	+	-	+	+	+	+
Trehalose	+	+	+	-	+	(+)	(+)	+

+ Denotes a positive reaction.

(+) Denotes a strong reaction but not complete.

~ Denotes a weak reaction.

- Denotes a negative reaction.

<sup>a</sup> Results are approximate average of two determinations performed on the same sample.

<sup>b</sup> Corresponds to *Lb. delbrueckii* ssp. *bulgaricus*.

No reaction was observed for L-arabinose, gluconate, inulin, mannitol, melizitose, melibiose, sorbitol, D-turanose, D-xylose, or L-xylose.

gave negative reaction. The *Lb. acidophilus* in the starter DV B-100 has also given some different results in comparison with the rest where, amigdaline,  $\beta$ -gentibiose and salicine were not metabolised for this strain, whilst most of the rest were able to metabolise them.

In contrast, *Lb. delbrueckii* ssp. *bulgaricus* strain tested has given a pattern of carbohydrate utilisation completely different from the data reported in Table 2.6. For example, the carbohydrate fermentation pattern for this species is able only to ferment few sugars such as fructose, glucose, and lactose. Nevertheless, the results showed a wider range of fermentation, and these included galactose, maltose, D-mannose, D-raffinose, sucrose, trehalose and N-acetyl glucosamine which may suggest that such isolate could be *Lb. delbrueckii* ssp. *lactis* (Stanley G., personal communication; see also Tamime, 1990).

#### 4.2.2.3 Enzymatic activities of streptococci

Some intermediate reactions were observed for phosphatase alkaline, and  $\alpha$ -galactosidase for the isolates for the isolates from starter cultures ABT-1 and MY 087, respectively (see Table 4.7). Thus, the profiles of enzymatic activities of the *Str. thermophilus* isolates are shown in Table 4.7, and the results reflect that the enzymatic activities of these tests generally were more consistent when compared with isolates of *Lb. acidophilus* and *Bifidobacterium* species. For example,  $\beta$ -galactosidase and leucine aminopeptidase activities were 3-5, and present in all of the isolated strains; however, most of the strains also showed weak activity for esterase C(4), naphthol-AS-BI-phosphohydrolase and phosphatase acid, but no activity for the latter enzyme was detected for the isolate from starter culture MSK 2 (see Table 4.7).

In order to confirm the results of API-ZYM test method, the rapid ID 32 STREP test method was also used and the results are shown in Table 4.8. The carbohydrates metabolism and enzymatic activities of the *Str. thermophilus* showed a very consistent pattern for all the isolates. Lactose and saccharose were fermented by all the isolates; however, slightly weaker reaction was detected for the isolate from

**Table 4.7** Some enzymatic activities (API-ZYM) of *Str. thermophilus* isolates from different starter cultures<sup>a</sup>.

Enzyme	ABT-1	ABT-3	MSK 2	MSK B2	DV B-100	MY 087
Phosphatase alkaline	1	0	0	0	0	0
Esterase (C <sub>4</sub> )	1	1	1	2	1	1
Esterase lipase (C <sub>8</sub> )	2	2	1	2	2	0
Leucine aminopeptidase	5	4	5	4	3	5
Valine aminopeptidase	1	0	1	0	0	0
Phosphatase acid	2	1	0	1	1	1
Naphtol-AS-BI-phosphohydrolase	1	1	1	1	1	1
$\alpha$ -galactosidase	0	0	0	0	0	1
$\beta$ -galactosidase	5	4	5	5	3	5

<sup>a</sup> Numbers 1 to 4 indicate intermediate reactions (5-30 nM), number 5 indicates maximum activity (40 nM) and zero indicates negative reaction.

Results are approximate average of two determinations performed on the same sample.

No activity was observed for lipase (C<sub>14</sub>), trypsin, chymotrypsin, cystine aminopeptidase,  $\beta$ -glucuronidase,  $\alpha$ -glucosidase,  $\beta$ -glucosidase, N-acetyl- $\beta$ -glucosaminidase,  $\alpha$ -mannosidase, or  $\alpha$ -fucosidase.

**Table 4.8** Carbohydrate metabolism and enzymatic activities (ID 32 STREP) of *Str. thermophilus* isolates<sup>a</sup>.

Carbohydrate/enzyme	ABT-1	ABT-3	MSK 2	MSK B2	DV B-100	MY 087
Lactose	+	(+)	+	+	+	+
Saccharose	+	(+)	+	+	(+)	+
$\beta$ -galactosidase	+	+	+	+	+	+
Alanine-phenylalanine-proline arylamidase	+	+	+	+	+	+
Urease	+	+	+	+	+	+

+ Represents positive reaction.

(+) Represents strong reaction but not complete.

<sup>a</sup> Results are approximate average of two determinations performed on the same sample.

No activity was found for ribose, mannitol, sorbitol, trehalose, raffinose, L-arabinose, D-arabitol, cyclo-dextrin, glycogen, pullulan, maltose, melibiose, melezitose, methyl-B-D-glucopyranoside, tagatose, arginine di-hydrolase,  $\beta$ -glucosidase,  $\beta$ -glucuronidase,  $\alpha$ -galactosidase, alkaline phosphatase, acetoin production,  $\beta$ -mannosidase, pyrrolglutamic acid arylamidase, N-acetyl- $\beta$ -glucosaminidase, or glycyl-tryptophane arylamidase.



starter culture ABT-3. The enzymatic activities on  $\beta$ -galactosidase, alanine-phenylalanine-proline arylamidase and urease showed positive reaction for all the strains isolated. When the data shown in Table 4.8 was compared with a computer programme supplied by the manufacturer, the output identified all the isolates as *Str. thermophilus*.

### 4.3 Conclusions

The growth of *Bifidobacterium* species was enormously enhanced when cysteine was present in the enumeration medium. The presence of cysteine only affected the size of the colonies rather than the number of colonies. The recovery counts of the micro-organisms of the starter cultures were similar to the supplier's specifications with the exception of the starter culture MSK 2 which had a count 1 fold less count for each *Bifidobacterium* and *Str. thermophilus* species, respectively.

Although morphological studies had limitations, the results illustrated at a genera level that the isolates belonged to bifidobacteria, lactobacilli and streptococci. The possible suggestion that *Bifidobacterium* spp. isolates from starter cultures AB, ABT-1, ABT-3 and MSK B2 belong to *Bif. animalis* require further physiological tests to confirm such species; however, the enzymatic activities tests failed to confirm such observations.

The enzymatic activities test methods (API-ZYM and API 50 CHL) of bifidobacteria isolates profiled a wide range of enzymes which helped, in part, to identify/confirm some isolates. In general, isolates from starter cultures MSK 2 (*Bif. longum*), AC/BL (*Bif. infantis*) and DV B-100 (*Bif. bifidum*) have been confirmed at species level, and the other bifidobacteria isolates could be considered closely related, but could not be identified at species level. However, the ID 32A test method was specific for the identification of *Bif. adolescentis* on species level, but not the other bifidobacteria, and the results obtained confirmed that unknown bifidobacteria isolates from starter cultures AB, ABT-1, ABT-3 and MSK B2 do not belong to such species.

Similar confirmations were also observed for *Lb. acidophilus* and *Str. thermophilus* isolates. However, with regards to *Lb. delbrueckii* ssp. *bulgaricus* isolates from starter culture MY 087, it is more likely to be *Lb. delbrueckii* ssp. *lactis* because it was able to ferment more sugars when compared with *Lb. delbrueckii* ssp. *bulgaricus*.

## **CHAPTER FIVE**

### **PRODUCTION OF SET-TYPE FERMENTED MILKS USING BIFIDOBACTERIA**

## **CHAPTER FIVE: PRODUCTION OF SET-TYPE FERMENTED MILKS USING BIFIDOBACTERIA**

### **5.1 Preliminary Studies**

This section describes some aspects of the compositional and microbiological quality control assessments performed on all of the raw materials employed for the manufacture of the set-type fermented milks in the present study.

#### **5.1.1 Quality of the skimmed milk powder (SMP)**

In order to standardise the quality of the milk base throughout the achievement of this study, SMP was used as the basic material for the manufacture of set-type fermented milks. Therefore, the factors, which can influence (see section 2.4.1) the compositional quality of fresh milk, were minimised.

The specifications (*i.e.* compositional, physical and microbiological) of SMP for recombination plays an important role on the quality of the final product. The analysis of SMP were carried out according to the methods described in section 3.4, and Table 5.1 shows the compositional, physical and microbiological qualities of SMP. The results agreed with the specifications reported by Sjollem (1988), and consequently, the milk was suitable for yoghurt making. The heat number of the SMP was 80 which classified the product as a 'medium heat' suitable for the production of fermented milk products (Wilcek, 1990).

#### **5.1.2 Quality of milk protein concentrate (MPC)**

As mentioned in section 3.1.3, MPC was used to fortify the protein content in the milk base. The MPC was prepared from fresh milk by means of ultrafiltration and its lactose content was reduced by means of enzymatic hydrolysis. The use of MPC allowed a great level of flexibility with regard to the fortification of the protein content during the formulation in the preliminary trials.

Table 5.1 Chemical, compositional and microbiological qualities of SMP, MPC and AMF

Test	SMP	MPC	Sjollema (1988) <sup>a</sup>	AMF	Sjollema (1988) <sup>b</sup>
Protein (g 100g <sup>-1</sup> )	36.42	81.01	-	-	-
Fat (g 100g <sup>-1</sup> )	0.57	1.64	max. 1.0	99.87	min. 99.9
Moisture (g 100g <sup>-1</sup> )	3.38	5.04	max. 4.0	0.13	max. 0.1
Ash (g 100g <sup>-1</sup> )	8.10	7.59	-	-	-
Lactose (%)	51.52 <sup>c</sup>	4.71 <sup>d</sup>	-	-	-
Titratable acidity (%)	0.14	0.13	max. 0.15	-	-
Peroxide value (meq O <sub>2</sub> kg <sup>-1</sup> )	-	-	-	0.16	max. 0.20
Scorched particles	grade A	grade A	min. grade B	-	-
Insolubility index	<0.1	<0.1	max. 0.5	-	-
Heat number	80	-	-	-	-
Antibiotics	-ve	-ve	-	-	-
Total viable count (cfu g <sup>-1</sup> )	8.7x10 <sup>3</sup>	4.0x10 <sup>2</sup>	max. 5.0x10 <sup>4</sup>	30	max. 100
Plate count 55 °C (cfu g <sup>-1</sup> )	4.4x10 <sup>2</sup>	<10	max. 1.0x10 <sup>4</sup>	<10	-
<i>Enterobacteriaceae</i> (cfu g <sup>-1</sup> )	<10	<10	max. 10	<10	max. 10
Yeasts and moulds (cfu g <sup>-1</sup> )	<10	<10	max. 100	<10	max. 10
Lipolytic bacteria (cfu g <sup>-1</sup> )	-	-	-	<10	-

<sup>a</sup> Specifications for SMP recommended by Sjollema (1988).<sup>b</sup> Specifications for AMF recommended by Sjollema (1988).<sup>c</sup> Lactose was calculated by difference.<sup>d</sup> Value calculated by difference and corresponds to lactose and other carbohydrates.

Most of the quality assurance tests performed were similar to those mentioned above for SMP, and the results are also shown in Table 5.1. The protein level is very high ( $\sim 81\text{g } 100\text{g}^{-1}$ ) and the lactose content is very low ( $\sim 4.8\text{g } 100\text{g}^{-1}$ ).

### **5.1.3 Quality of anhydrous milk fat (AMF)**

Anhydrous milk fat was used in the recombination process to manufacture the fermented milk products. Table 5.1 shows the compositional and microbiological qualities of AMF, and the results are similar to the specifications recommended by Sjollena (1988) and Tamime and Kirkegaard (1991). No lipolytic bacteria were present in the AMF at  $10^{-1}$  dilution which demonstrates that the product was likely to be free from soapy-flavours.

### **5.1.4 Starter cultures**

Section 4.1 describes in detail the enumeration of the starter cultures, and Table 4.1 shows the recovery values.

## **5.2 Production of Set-Type Fermented Milks using Single Strains of Bifidobacteria**

Given the strong suggestions of beneficial effects associated with the consumption of fermented milk products containing bifidobacteria could lead to the human health (see sections 2.7.4 to 2.7.8), it was decided to carry out some experimental trials to produce fermented milk products by using single strains of bifidobacteria from human origin.

### **5.2.1 Single strains from the National Collection of Food Bacteria (NCFB)**

As mentioned in section 3.1.4.1, *Bif. adolescentis* 2230, *Bif. bifidum* 2715, *Bif. breve* 2258, *Bif. infantis* 2205 and *Bif. longum* 2259 were obtained from the NCFB and were used to ferment the milk. The 2230 and 2715 cultures were maintained in MRS

broth (Oxoid, Ltd.) to which cysteine ( $0.05\text{g } 100\text{g}^{-1}$ ) was added. The 2258 and 2259 cultures were maintained in RCM broth (Reinforced Clostridial Medium, Oxoid Ltd.) containing cysteine ( $0.05\text{g } 100\text{g}^{-1}$ ) and the 2205 culture was maintained into MRS broth (Oxoid, Ltd.). Overnight cultures grown for ~16 h in these media were used as inocula at a rate of  $2\text{ml } 100\text{ml}^{-1}$  to ferment the milk.

The production of set-fermented milks was carried out following the stages shown in Fig. 2.2. The total solids of the milk was approximately  $14.5\text{g } 100\text{g}^{-1}$ , of which  $5.0\text{g}$  was protein,  $6.9\text{g}$  was lactose and  $1.5\text{g}$  was fat. The incubation period was 18 h at  $\sim 40^{\circ}\text{C}$ .

The quality of the products did not satisfy the minimum requirements. The pH values were at best border line (*i.e.* between 4.8 to 5.4), and there was excessive whey formation and the coagulum was extremely weak. Therefore, it was decided to modify the composition of the milk base. The protein content was increased by using MPC, and the approximate composition of the milk base was: total solids  $\sim 15.5\text{g } 100\text{g}^{-1}$  of which proteins  $7.5\text{g}$ , fat  $1.5\text{g}$ , and lactose  $5.4\text{g}$ . The results obtained were also unsuccessful. The characteristics of the products were similar to the previous trials, excessive whey syneresis, the coagulum was very weak and in some cases unpleasant and/or 'cheesy' odour. As a consequence of such observations, commercial single strains of *Bifidobacterium* species were used for the production of set-type fermented milks of an acceptable quality.

### 5.2.2 Commercial single strains

The commercial single strains cited in section 3.1.4.1 were also used to produce fermented milks using single strains of bifidobacteria, and in order to enhance the growth of *Bifidobacterium* species, natural oligosaccharides [*i.e.* Jerusalem Artichoke flour and Raftiline (see section 3.1.3)] were added to the milk base (Tamime *et al.*, 1995) at a rate of  $1\text{g } 100\text{g}^{-1}$ . The approximate composition of the milk base was as follow: total solids  $\sim 16.5\text{g } 100\text{g}^{-1}$  of which  $7.6\text{g}$  of proteins,  $1.5\text{g}$  of fat and  $6.1\text{g}$  of lactose. Table 5.2 illustrates the pH values obtained after 24 h of incubation.

**Table 5.2** The pH values of fermented milks after 24 h of incubation period using commercial single cultures of bifidobacteria<sup>a</sup>

Code	<i>Bif. infantis</i>		<i>Bif. bifidum</i>		<i>Bif. longum</i>			
	410		BB 11	BL	BBL	BB 46	2	913
Milk	5.91		5.36	4.98	4.65	5.18	4.64 <sup>b</sup>	5.04
Observations	ES		ES	ES	ES	NS	ES	ES
Milk+Rafiline	5.02		5.36	5.00	4.91	5.31	4.96	5.11
Observations	ES		ES	ES	ES	NS	ES	ES
Milk+J. Artichoke	5.63		5.29	4.93	4.79	5.31	4.75	5.09
Observations	EW		ES	ES	ES	NS	ES	ES

<sup>a</sup> The average range of pH of the milk bases were: (a) milk ~6.64, (b) milk ÷ Raftiline ~6.61, and (c) milk + J. Artichoke ~6.61.

<sup>b</sup> The incubation period was only 18 h.

NS No syneresis.

ES Excessive syneresis.

EW Extremely weak coagulum.



The gel of the products were quite firm. However, with the exception of the *Bif. longum* BB46, most of the products had excessive whey syneresis. In spite of 24 h incubation period most of the strains did not acidify the milk base to acceptable values (*i.e.* < pH 5) with the exception of *Bif. longum* BBL and 2 (Table 5.2). The fermented milks manufactured by adding Raftiline and Jerusalem Artichoke flour did not enhance the growth of the micro-organisms, and in addition the latter additive precipitated to the bottom of the plastic cups during the incubation period. Thus, the results obtained in these preliminary trials using single strains of bifidobacteria (NCFB) and commercial were not satisfactory, and as consequence it was decided to use commercial mixed starter cultures containing different *Bifidobacterium* species, and lactic acid bacteria.

### **5.3 Production of Set-Type Fermented Milks using Commercial Mixed Starter Cultures Containing Bifidobacteria**

A total of three trials, *i.e.* 24 batches, of set-type fermented milks were produced as described in section 3.3 and as illustrated in Figure 2.2. Seven different starter cultures (see Table 3.10) containing bifidobacteria and one yoghurt starter culture were used.

#### **5.3.1 Chemical composition of the milk base**

The average chemical composition of twenty four batches of the milk base is shown in Table 5.3, and individual trials' results are shown in Appendix VI. The total solids of the milk base ranged between 14.4 and 14.6g 100g<sup>-1</sup> (SED= 0.04), and the protein content ranged between 5.4 and 5.6g 100g<sup>-1</sup> (SED= 0.015). Such small variations could be attributed to the normal routine of operational factors. No significant variations were observed in the fat and ash contents, and pH values. However, small variations were observed in the lactose content (~6.23 to 6.57 g 100g<sup>-1</sup>).

**Table 5.3** Compositional quality (g 100g<sup>-1</sup>) of the milk base and fermented milks (fresh and stored)<sup>a</sup>.

	Protein	Fat	Ash	Total solids	pH
<b>Milk base</b>					
AB	5.45	1.46	1.09	14.46	6.46
AC/BL	5.50	1.43	1.09	14.51	6.48
DV B-100	5.46	1.44	1.10	14.45	6.50
MSK 2	5.49	1.45	1.10	14.52	6.48
ABT-1	5.51	1.46	1.09	14.47	6.47
ABT-3	5.46	1.42	1.09	14.43	6.47
MSK B2	5.48	1.44	1.08	14.49	6.48
MY 087	5.51	1.45	1.09	14.49	6.48
<b>Fresh</b>					
AB	5.50	1.46	1.09	14.15	4.37
AC/BL	5.54	1.46	1.09	14.09	4.46
DV B-100	5.52	1.45	1.08	14.17	4.52
MSK 2	5.55	1.46	1.09	14.29	4.68
ABT-1	5.55	1.46	1.10	14.19	4.59
ABT-3	5.54	1.47	1.08	14.06	4.51
MSK B2	5.53	1.47	1.09	14.13	4.53
MY 087	5.54	1.46	1.09	14.20	4.54
<b>Stored</b>					
AB	5.50	1.43	1.10	14.16	4.16
AC/BL	5.52	1.46	1.10	14.14	4.29
DV B-100	5.49	1.44	1.10	14.15	4.32
MSK 2	5.52	1.45	1.10	14.25	4.63
ABT-1	5.52	1.44	1.10	14.10	4.47
ABT-3	5.50	1.42	1.09	14.11	4.41
MSK B2	5.53	1.43	1.10	14.11	4.27
MY 087	5.52	1.46	1.09	14.15	4.31
SED <sup>b</sup>	0.015	0.012	0.007	0.04	

<sup>a</sup> Results are average of a single sample analysed in duplicate in each of the three trials.

<sup>b</sup> Standard Error of the Differences.

### 5.3.2 Whey protein denaturation

The degree of whey protein denaturation [*i.e.* Immunoglobulins (Ig),  $\alpha$ -lactalbumin ( $\alpha$ -La), serum albumin lactoferrin (Sa/Lf) and  $\beta$ -lactoglobulin ( $\beta$ -Lg)] was determined as described in section 3.9.6. Table 5.4 shows the results of the whey protein denaturation expressed as percentage, and Appendix VII illustrates the results of each trial.

The average degree of denaturation of whey protein fractions expressed as a percentage in regression order could be summarised as follows: Ig (49),  $\alpha$ -La (70), Sa/Lf (76) and  $\beta$ -Lg (90). The denaturation of  $\beta$ -Lg and  $\alpha$ -La play a major physico-chemical role towards the firmness of fermented milk products (Robinson and Tamime, 1993), and these results confirm such observation in particular the interaction between the *K*-casein and denatured  $\beta$ -Lg and  $\alpha$ -La (Mottar *et al.*, 1987, 1989).

Analysis of variance performed on the data of protein denaturation of the eight samples indicated that no statistically significant differences were detected ( $P > 0.05$ ) between them with the exception of Sa/Lf component that showed significant differences ( $P < 0.05$ ). This suggests that a good replication was carried out during the heat treatment of each batch.

### 5.3.3 Compositional quality of fermented milks (fresh and stored)

The average compositional quality of twenty four fermented milks (fresh and stored) is shown in Table 5.3, while the individual trials' results are illustrated in Appendix VIII. The protein, fat and ash contents were similar to the milk bases (see Table 5.3); however slight variations were observed in the total solids contents, that could be attributed to the metabolic activity of the starter cultures which could have utilised 30 to 40% of the lactose content (Barrantes *et al.*, 1994). Nevertheless, analysis of variance showed significant differences to these constituents when compared with the milk bases ( $p < 0.001$ ). No significant differences were observed for the fermented milks (*i.e.* fresh and stored) during the storage period.

**Table 5.4** Denaturation of whey protein during the heat treatment<sup>a</sup>.

	Ig	Sa/Lf	$\beta$ -Lg	$\alpha$ -La
AB	45.7	72.5	89.7	69.7
AC/BL	45.8	72.6	89.4	66.8
DV B-100	57.0	79.4	90.9	72.3
MSK 2	48.6	77.1	90.1	69.5
ABT-1	48.6	75.9	89.7	69.2
ABT-3	53.2	77.1	90.6	72.3
MSK B2	51.4	79.5	90.1	70.4
MY 087	48.4	77.1	91.2	73.3
SED <sup>b</sup>	2.89	1.46	0.76	3.25

<sup>a</sup> Figures are expressed as a percentage of denaturation.<sup>b</sup> Standard Error of the Differences.

Ig Immunoglobulin.

 $\alpha$ -La  $\alpha$ -lactoalbumin.

Sa/Lf Serum albumin lactoferrin.

 $\beta$ -Lg  $\beta$ -lactoglobulin.

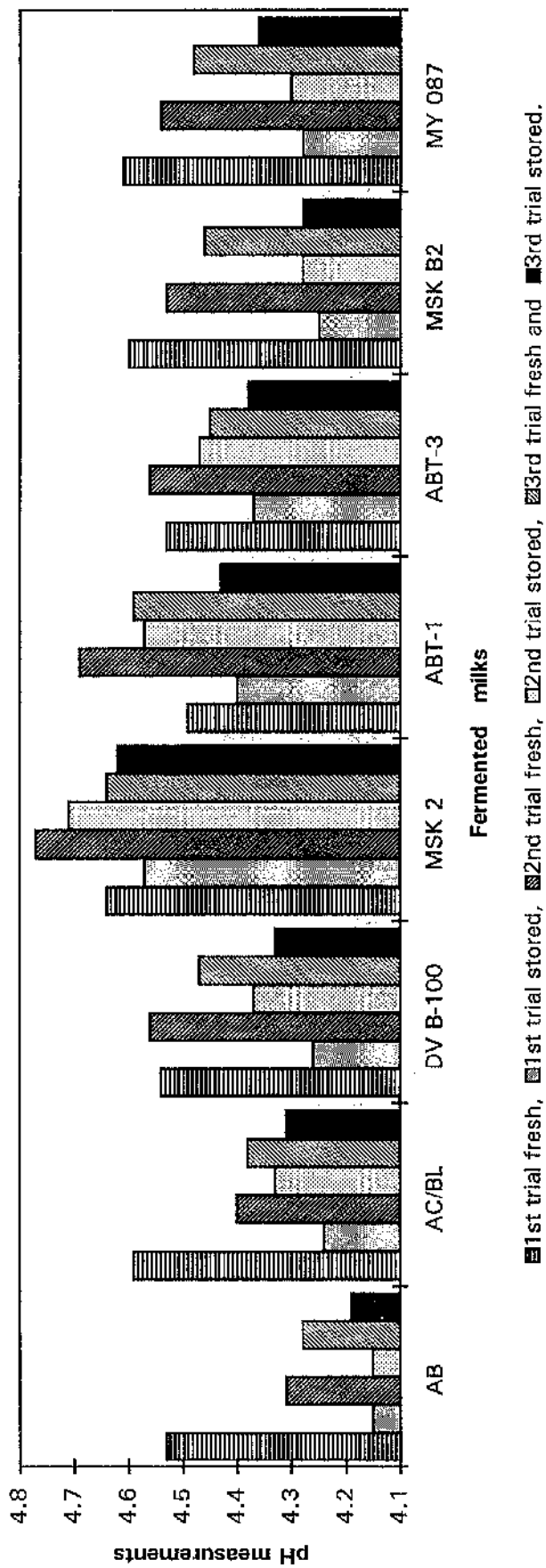
### 5.3.3 Acidification of the milk base and post-acidification of the fermented milks

The starter cultures used contained different species of micro-organisms, and as a result the incubation period required to reach the desired level of acidification varied. For example, for the milk base inoculated with starter cultures DV B-100, ABT-3, MSK B2 and MY 087, the incubation period were between 6 and 10 hours; while the other starter cultures (*i.e.* AB, AC/BL, MSK 2 and ABT-1) required 13 to 22 h. Incidentally, the incubation temperature was  $\sim 40^{\circ}\text{C}$ .

The pH values of the milk base averaged around 6.48 see Appendix VI, and the pHs of the fermented milks (fresh and stored) are shown in Appendix VIII. The decrease in pH measurements of the fermented milks (fresh and stored) is illustrated in Figure 5.1 where the highest acidification rate was observed for starter culture AB whilst the lowest was for MSK 2. For example, in the first trial the pHs of fresh and stored products ranged between 4.49 to 4.64 and 4.15 to 4.57, respectively. A similar pattern was also observed in the second and third trials. The mean pH decrease during 20 days storage of all the products was  $\sim 0.17$  units. However, the highest individual mean pH decrease  $\sim 0.26$  during the storage period was for starter culture ABT-1, and the lowest was for starter culture MSK 2  $\sim 0.05$  units. This showed the tendency of post acidification in fermented milks during the storage period due to the continued metabolic activity of the starter cultures (Tamime *et al.*, 1987; Becker and Puhani, 1989).

### 5.3.4 Analysis of organic acids

Organic acids in the manufacture of fermented milks, contribute towards the refreshing taste and flavour of the product. In addition to the production of lactic acid during the fermentation process, other organic acids such as acetic, formic, orotic, citric and butyric are also produced. According to Rasic and Kurmann (1978) and Tamime and Robinson (1985) the type of organic acids in the product provides relevant data with regard to the metabolic activity of the starter culture.



**Figure 5.1** The rate of acidification and the influence of storage of fermented milks after 20 days at 5 °C.

Results are average of two determinations performed on the same sample.

The average content of organic acids content ( $\mu\text{g g}^{-1}$ ) in the milk bases and fermented milks (fresh and stored) is shown in Table 5.5, while Figures 5.2 and 5.3 show the level of acetic and lactic acids produced, respectively. Appendix IX shows the results of each individual trial. As it was expected the pattern of the organic acids content were mainly influenced by the metabolic activity of each individual starter culture used. A generalised feature could be observed where there was a remarkable increase in lactic and acetic acids contents in all of the products when compared with the milk bases (see Figures 5.2 and 5.3). The largest amount of acetic acid was produced by starters cultures AB and AC/BL when compared with the rest.

There are some data available on the organic acids content of yoghurt that has been reported by Marsili *et al.* (1981), Bevilacqua and Califano (1989) and Laye *et al.* (1993), but no data is available on fermented milk products made with starter cultures containing bifidobacteria and *Lb. acidophilus*. In the present study the following observations could be made:

- Orotic and citric acids had been utilised by all the starter cultures;
- Pyruvic acid was increased, and the major increase was observed for starter cultures AB and AC/BL fermented milk products;
- There was a remarkable and variable increase of lactic and acetic acids, and higher amounts of the latter was observed in fermented milks made with starter cultures AB and AC/BL;
- Uric/formic acids had increased slightly and remained constant during the storage period;
- Hippuric acid was utilised slightly, but complete hydrolysis was observed for starter culture MSK 2.
- Propionic and butyric acids were not detected in the milk bases or in any of the products.

With the exception of starter cultures AB and AC/BL (*i.e.* had the highest acetic acid content), all the products showed similar patterns when compared with yoghurt which also showed similar pattern to those reported in the literature (Rasic

**Table 5.5** Means of organic acids content ( $\mu\text{g g}^{-1}$ ) of milk bases, and fermented milks (fresh and stored)<sup>a</sup>.

	Orotic	Citric	Pyruvic	Uric/formic	Hippuric
<b>Milk base</b>					
AB	102	1065	11	20	32
AC/BL	110	1120	6	22	34
DV B-100	112	1152	6	24	34
MSK 2	116	1183	6	24	32
ABT-1	109	1153	13	22	36
ABT-3	107	1188	11	22	35
MSK B2	117	1417	8	24	37
MY 087	115	1285	13	25	37
<b>Fresh</b>					
AB	76	912	102	29	28
AC/BL	85	893	124	30	28
DV B-100	92	842	52	26	32
MSK 2	91	726	83	30	2
ABT-1	86	727	73	30	24
ABT-3	92	786	64	31	30
MSK B2	90	698	68	30	28
MY 087	93	801	53	30	30
<b>Stored</b>					
AB	70	804	107	29	29
AC/BL	81	823	127	31	28
DV B-100	91	766	44	34	30
MSK 2	88	675	67	29	1
ABT-1	85	687	81	29	25
ABT-3	93	714	49	28	30
MSK B2	87	637	66	29	25
MY 087	89	748	34	31	28

<sup>a</sup> Results are average of a single sample analysed in duplicate in each of the three trials.



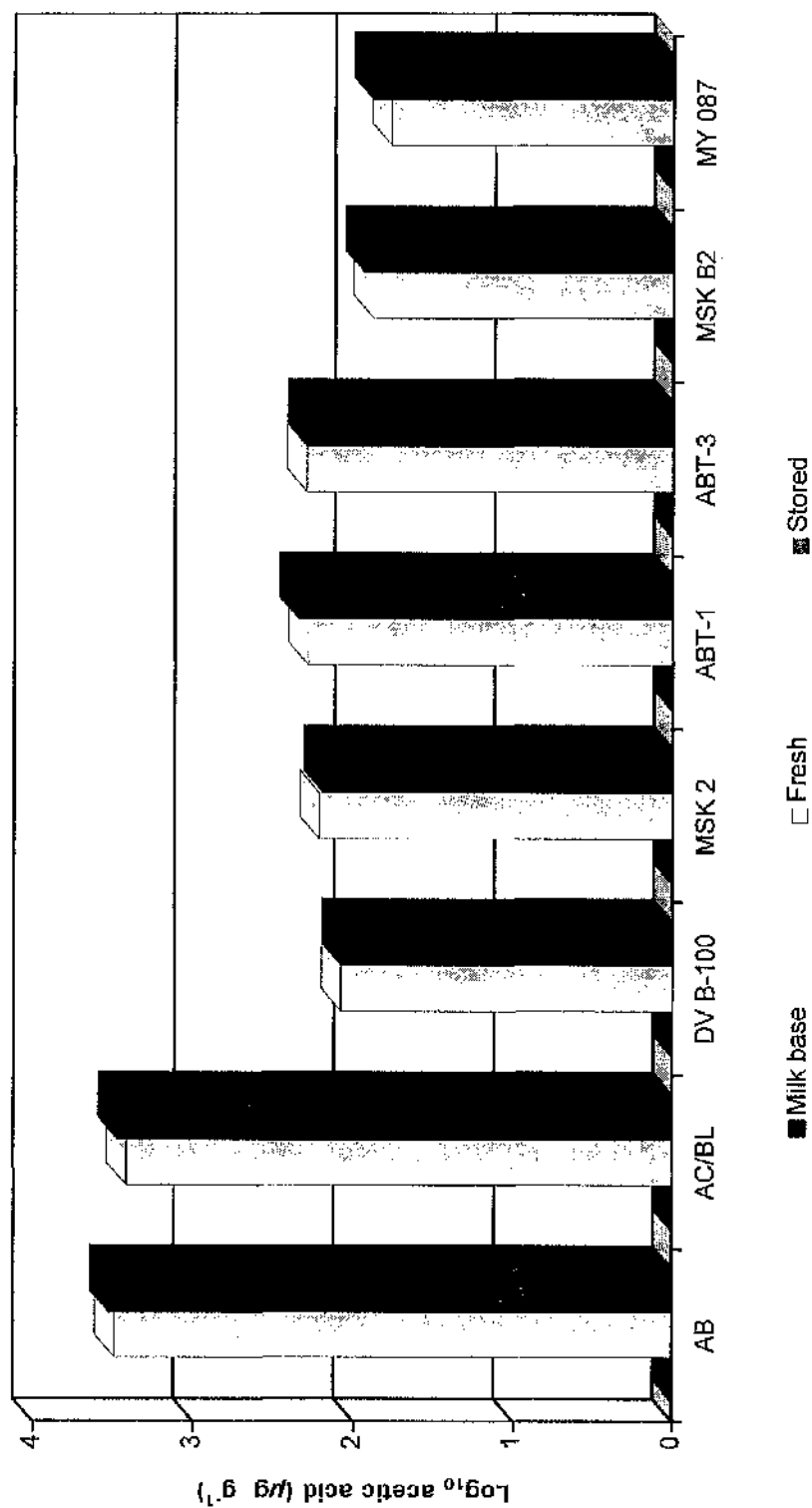
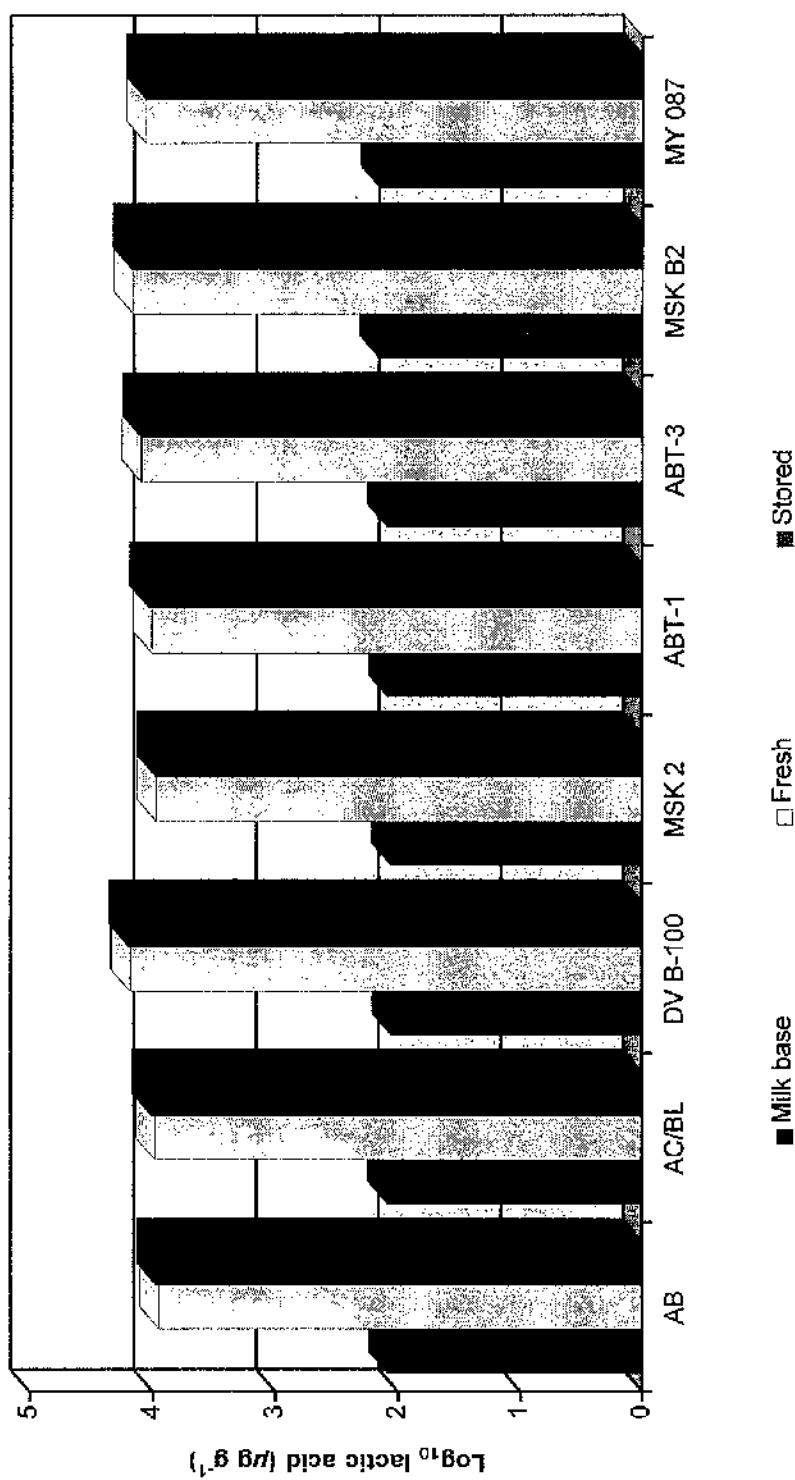


Figure 5.2 Acetic acid content ( $\mu\text{g g}^{-1}$ ) of different milk bases and fermented milks (fresh and stored)<sup>a</sup>.

<sup>a</sup> Results are average of three trials and two determinations performed on the same sample.



**Figure 5.3** Lactic acid content ( $\mu\text{g g}^{-1}$ ) of different milk bases and fermented milks (fresh and stored)<sup>a</sup>

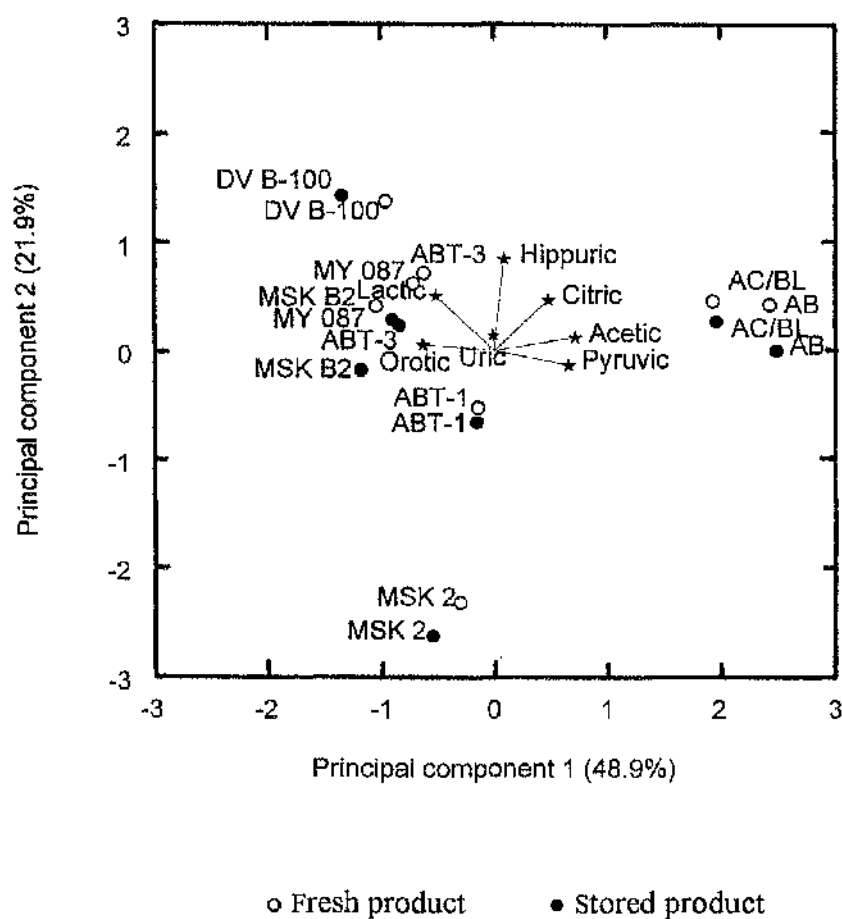
<sup>a</sup> Results are average of three trials and two determinations performed on the same sample.

and Kurmann, 1978; Marsilli *et al.*, 1981; Laye *et al.*, 1993). The higher acetic acid content of AB and AC/BL products could be attributed to the metabolic activity of *Bifidobacterium* species.

Analysis of variance performed on the organic acids contents showed high statistical significant differences ( $P < 0.001$ ) between the fermented milks. This could be attributed to the different metabolic activities of the starter cultures. Incidentally, citric, lactic, and acetic acids of the fermented milks fresh and stored showed statistical significant differences which ranged between  $P > 0.01$  and  $P < 0.05$ .

Principal Component Analysis (PCA) was performed on the correlation matrix of the organic acids content of fermented milks (fresh and stored.). The first two PCs accounted for 70.8 % of the variation ( $PC1 = 48.9\%$  and  $PC2 = 21.9\%$ ). A Principal Component bi-plot was produced for the data (see Figure 5.4), and within the bi-plot samples which are clustered together are similar. The angles between the vectors representing the variables (*i.e.* orotic, citric, lactic, acetic, and hippuric acids) are approximations to the correlations between each variable. Hence, variables with a small angle between them are positively correlated, and those with a large angle ( $180^\circ$ ) are negatively correlated, and those at right angles ( $90^\circ$ ) are uncorrelated. Samples with high loadings on each vector have relatively high amount of that variable. The accuracy with which the bi-plot reproduces the relationships between variables is a function of the percentage variance explained. Thus, Figure 5.4 shows 70.8% of the variance, and is a good representation of the data.

Several clusters were observed, for example, the fermented milk made with starter culture MSK 2 was notably low in most of the organic acids, and the fermented milks (AB and AC/BL) showed high content of citric and acetic acids; however, the same products were relatively lower in lactic and orotic acids content (Figure 5.4). The opposite was observed for the last cluster of points (*i.e.* DV B-100, ABT-3, MSK B2 and MY 087 fermented milks), and the fermented milk made with starter culture ABT-1 was average.



**Figure 5.4** Principal Components biplot of organic acids content using correlation matrix.

### 5.3.5 Microbiological quality of fermented milk products

The results of the microbiological quality control assessments performed on all of the products when fresh and stored are shown in Table 5.6. The total count of non-lactic acid bacteria in all of the samples tested were within a margin of acceptability *i.e.*  $<100$  cfu g<sup>-1</sup> (Tamime *et al.*, 1987). The coliforms, yeasts and moulds counts in the fermented milks (fresh and stored) were low  $<10$  cfu g<sup>-1</sup>, *i.e.* absence of such organisms at 10<sup>-1</sup> dilution with the exception of two products made with starter cultures MSK 2 and ABT-3. This could be attributed mainly to contamination during processing. The overall results demonstrate that all of the fermented milks, were produced under good hygienic standards and sanitary conditions.

### 5.3.6 Enumeration of starter culture organisms

One of the main factors, which is accounted for when using 'probiotic' organisms, is the viability of the starter culture during the storage period. Thus, growth and survival of these micro-organisms were examined and monitored in the milk base and in the products (fresh and stored), and the results are shown in Figure 5.5. Appendix X shows the enumeration results of each individual trial.

The growth pattern of *Bifidobacterium* species in the milk base during the fermentation period was heterogeneous and generally poor except when using starter cultures AB and AC/BL (Figure 5.5). In both products the viable counts had increased by 2 log<sub>10</sub> folds, and a slight reduction was observed after storing the fermented milks for 20 days at 5 °C. Nevertheless, the viable cells counts were well above 10<sup>6</sup> cfu g<sup>-1</sup> in the product at the time of consumption which complies with the efficacy of 'Bio' product (Robinson, 1989; Tamime *et al.*, 1995).

The viable cells counts of bifidobacteria in the milk base and fermented products (fresh and stored) remained constant (*i.e.* 10<sup>5</sup> - 10<sup>6</sup> cfu g<sup>-1</sup>) when using starter cultures DV B-100, ABT-3 and MSK B2 suggesting that these *Bifidobacterium* species do not grow well in milk and had tolerated the acidic

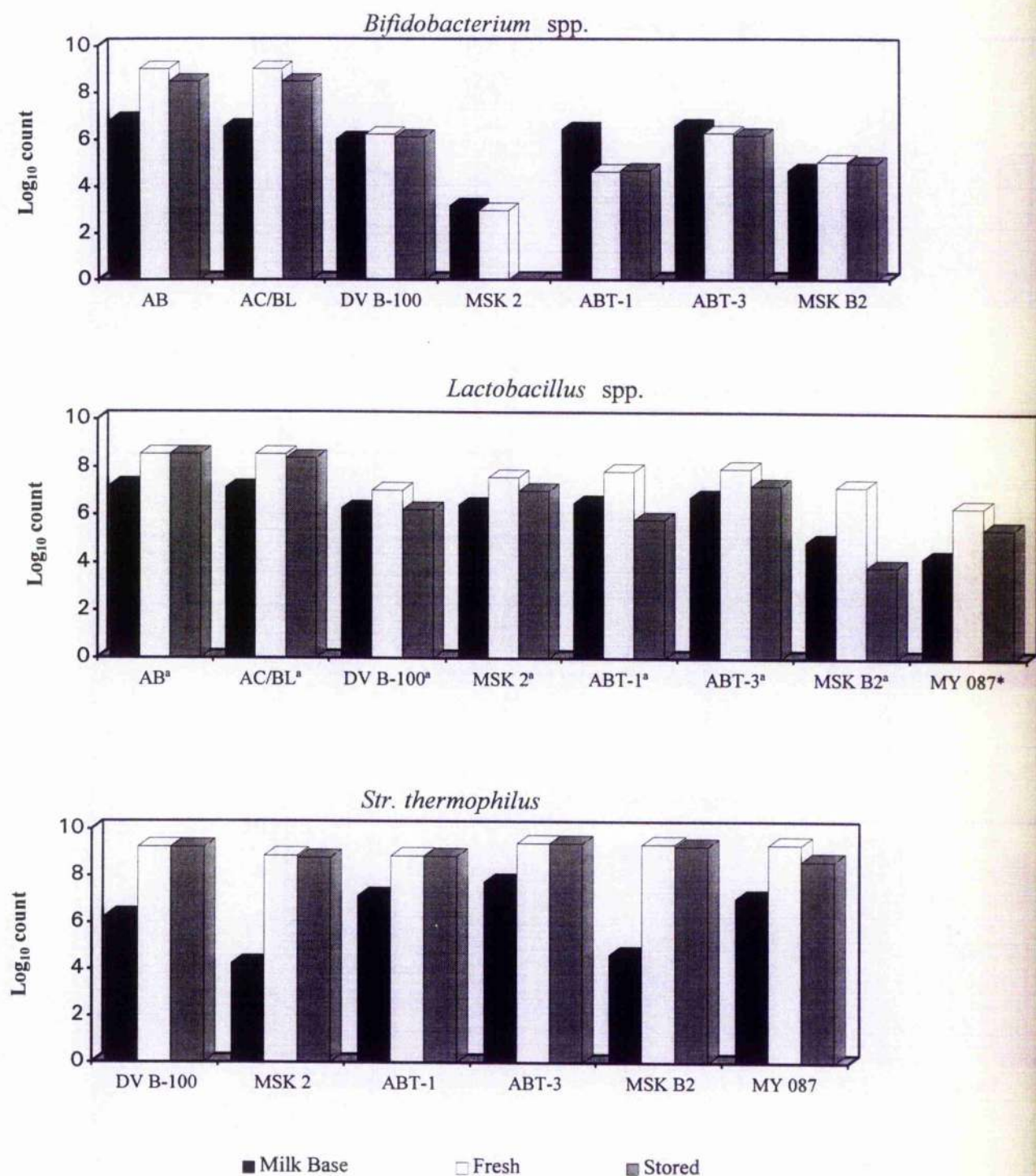
Table 5.6 Microbiological quality (cfu g<sup>-1</sup>) of different fermented milks.

	Fresh			Stored		
	Total count	Coliforms	Yeasts & moulds	Total count	Coliforms	Yeasts and moulds
<b>First trial</b>						
AB	<100 <sup>a</sup>	<10 <sup>b</sup>	<10	<100	<10	<10
AC/BL	<100	<10	<10	<100	<10	<10
DV B-100	<100	<10	<10	<100	<10	<10
MSK 2	1.5x10 <sup>3</sup>	<10	1.4x10 <sup>3</sup>	C	<10	C
ABT-1	<100	<10	<10	<100	<10	<10
ABT-3	<100	<10	3.2x10 <sup>2</sup>	<100	<10	C
MSK B2	<100	<10	<10	<100	<10	<10
MY 087	<100	<10	<10	<100	<10	<10
<b>Second trial</b>						
AB	<100	<10	<10	<100	<10	<10
AC/BL	<100	<10	<10	<100	<10	<10
DV B-100	<100	<10	<10	<100	<10	<10
MSK 2	<100	<10	<10	<100	<10	<10
ABT-1	<100	<10	<10	<100	<10	<10
ABT-3	<100	<10	<10	<100	<10	<10
MSK B2	<100	<10	<100 1 <100	<100	<10	<10
MY 087	1.7x10 <sup>3</sup>	<10	<10	<100	<10	<10
<b>Third trial</b>						
AB	<100	<10	<10	<100	<10	<10
AC/BL	<100	<10	<10	<100	<10	<10
DV B-100	<100	<10	<10	<100	<10	<10
MSK 2	<100	<10	<10	<100	<10	<10
ABT-1	<100	<10	<10	<100	<10	<10
ABT-3	<100	<10	<10	<100	<10	<10
MSK B2	<100	<10	<10	<100	<10	<10
MY 087	3.0x10 <sup>4</sup>	<10	<10	<100	<10	<10

<sup>a</sup> No growth at 10<sup>-2</sup> dilution.<sup>b</sup> No growth at 10<sup>-1</sup> dilution.

C Denotes contamination of the product.

Results are average of two determinations performed on the same sample.



**Figure 5.5** The enumeration of starter cultures (cfu g<sup>-1</sup>) during the production and storage of different fermented milks.

<sup>a</sup> Corresponds to *Lb. acidophilus*.

\* Corresponds to *Lb. delbrueckii* ssp. *bulgaricus*.

Results are average of three trials.

condition of the product during the storage period; however such counts may well be considered satisfactory (Robinson, 1989). A reduction in the viable cells of bifidobacteria was evident when using starter culture ABT-1, and no viable cells were recovered from the stored product made with starter culture MSK 2; such pattern of recovery of bifidobacteria in fermented milk may suggest the following:

- Some strains of *Bifidobacterium* species do not grow in milk or have restricted growth in the presence of *Str. thermophilus* and/or acidic condition (e.g. starter cultures DV B-100, ABT-3 and MSK B2)(Klaver *et al.*, 1993; Samona and Robinson, 1994);
- Most of the starter cultures exhibited acid tolerance with the exception of starter culture MSK 2;
- Growth of certain species of bifidobacteria in milk (e.g. AB and AC/BL) could be attributed to: (a) selected strains capable to grow in milk and (b) the possible associative growth in the presence of *Lb. acidophilus* (Klaver *et al.*, 1993).

All the starter cultures used showed an appreciable growth of *Lb. acidophilus* in milk where the viable cells counts had increased between 1 and 2  $\log_{10}$  folds (Figure 5.4). After the storage period of 20 days at 5°C, the pattern of recovery of viable cells of *Lb. acidophilus* in fermented milks made with different starter cultures could be summarised as follows: (a) the count remained constant (i.e. AB and AC/BL), (b) slight reduction (i.e. DV B-100, MSK 2 and ABT-3) and (c) a reduction by  $\sim 2 \log_{10}$  folds for products made with starter cultures ABT-1 and MSK B2. Most of the products contained viable cells  $>10^6$  cfu g<sup>-1</sup> with the exception of starter culture MSK B2, and these results confirm most of the fermented milks contained a satisfactory counts for a 'Bio' product (Robinson, 1989; Tamime *et al.*, 1995).

The *Lb. delbrueckii* ssp. *bulgaricus* viable cells count in yoghurt (fresh) made with starter culture MY 087 showed and increase by  $\sim 2 \log_{10}$  fold with slight reduction after the storage period (Figure 5.4). However, the overall count was  $\sim 10^6$  cfu g<sup>-1</sup>. It is evident that such low count of lactobacilli ensures low post-acidification during the storage period of the product (Barrantes *et al.*, 1994)



The viable cells counts of *Str. thermophilus* in fermented milks made with starter cultures DV B-100, MSK 2, ABT-1, ABT-3, MSK B2 and MY 087 showed a remarkable increase during the fermentation process (Figure 5.4). The counts of such organism ranged between  $10^8$  and  $10^9$  cfu g<sup>-1</sup> after the fermentation period, and the most dramatic increase was for starter cultures MSK 2 and MSK B2 (*i.e.* by  $\sim 4 \log_{10}$  folds). In general, all the *Str. thermophilus* of the different starter cultures have maintained a viable cells counts after the storage period  $\sim 10^8$ -  $10^9$  cfu g<sup>-1</sup>; however, a slight decrease was observed for the starter culture MY 087. This remarkable growth in milk and survival of *Str. thermophilus* confirms other reports published by Barrantes *et al.* (1994) and Rybka and Kailasapathy (1995).

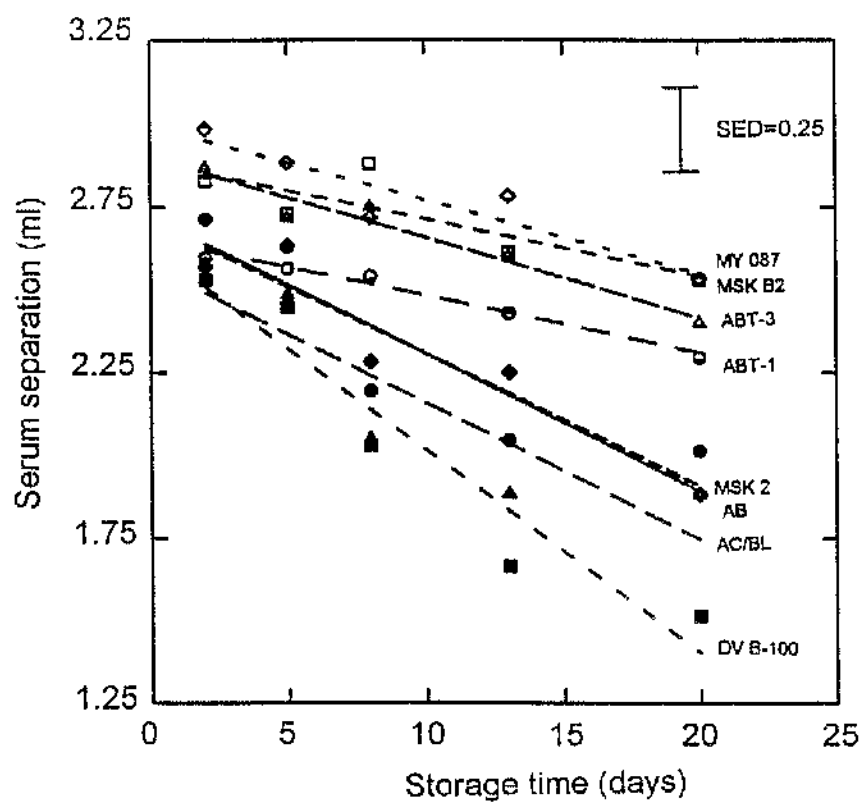
### 5.3.7 Rheological properties of fermented milks

The rheological properties of the eight different fermented milks were assessed by monitoring the rate of serum separation (syneresis) and firmness of the products as described in sections 3.9.4 and 3.9.5, respectively.

#### 5.3.7.1 Measurement of serum separation (syneresis)

The measurements of serum separation (syneresis) of eight different fermented milks are shown in Figure 5.6, and Appendix XI shows the results in each individual trial. After two days of storage at 5 °C, the fermented milks' syneresis values ranged between 2.5 and 3.0 ml 2h<sup>-1</sup>. During the storage period, the syneresis scores decreased to values between 1.5 and 2.5 ml 2h<sup>-1</sup> with the DV B-100 product had the minimum value, and MSK B2 and MY 087 had maximum values.

The development of syneresis over time was analysed using ANOVA with orthogonal polynomial contrasts for the time factor. The linear contrast measures a general increase or decrease. The quadratic contrast measures the concavity of the response. Higher order contrasts may be calculated, but are difficult to interpret.



**Figure 5.6** Changes in syneresis of fermented milks during 20 days storage at 5 °C. Results are average of three trials.

The analysis of variance of syneresis indicated a drop in the amount measured over time for all of the starter cultures ( $P < 0.001$ ). This decrease during the storage period was linear and the rate of decrease was dependent upon the starter culture used to produce the different fermented milks ( $P < 0.01$ ).

In general the serum separation (syneresis) of fermented milks with high initial syneresis decreased slower than those with a low initial syneresis. Good examples of the latter were the products made with starter cultures AC/BL, DV B-100 and MSK 2.

### **5.3.7.2 Measurement of firmness**

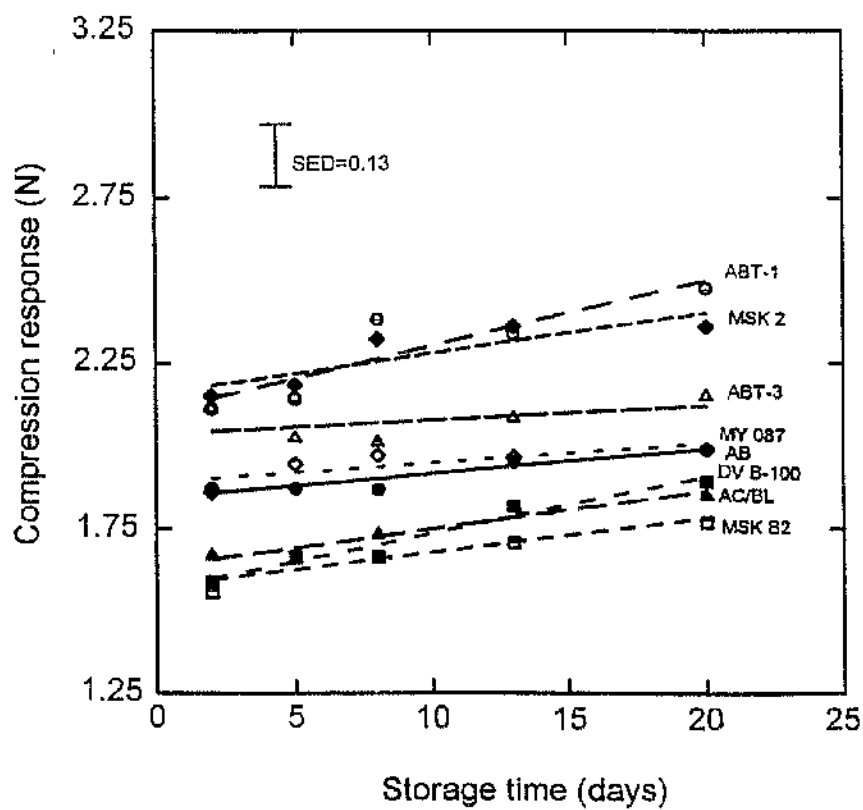
The results of the firmness measurements of eight different fermented milks are shown in Figure 5.7, and Appendix XII shows the results of each trial. After the two days of storage at 5 °C, the firmness measurements ranged between 1.6 and 2.2 N. During the storage period, the firmness of each fermented milk increased to values ranging between 1.8 and 2.5 after 20 days storage at 5 °C (Figure 5.7).

Analysis of variance with orthogonal polynomial contrasts was also performed on the measurements data of firmness of the fermented milks (see Figure 5.6). This analysis indicated an increase in the firmness over time for all of the samples ( $P < 0.001$ ), and the increase over the time period studied was linear; however, the rate of decrease was independent upon the starter culture used ( $P > 0.05$ ) to produce each fermented milk. Hence, in general the trend of increasing the firmness of the fermented milks was similar.

## **5.3.8 Organoleptic assessment**

### **5.3.8.1 Univariate Analysis**

The differences in individual attributes associated with starter cultures are shown in Table 5.7. While the means of starter culture x trial effects are illustrated in Appendix V.



**Figure 5.7** Changes in firmness of fermented milks during 20 days storage at 5 °C. Results are average of three trials.

**Table 5.7** Sensory attributes of plain, set-type fermented milks manufactured with different starter cultures. Mean values from 3 trials (6 replicate tastings within each trial - 2 x 3 time intervals.)

Attribute rating for culture effect (0 - 100)											
Culture	Smell			Flavour			Aftertaste		Physical		
	Acid/sour	Creamy	Sweet	Acid/sour	Creamy	Sweet	'Other'	Acid	Chalky	Viscosity	Serum
AB	48.9	19.0	7.7	69.1	17.9	6.8	30.1	62.6	17.7	15.9	72.0
AC/BL	43.3	24.9	9.4	54.7	36.5	10.3	23.5	48.1	21.3	15.8	71.5
DVB-100	28.3	34.8	15.5	43.4	43.7	11.6	8.9	35.1	25.0	20.1	74.1
MSK 2	27.5	28.2	12.3	31.7	40.2	15.5	11.5	23.6	27.9	21.6	70.8
ABT-1	30.3	35.3	16.8	42.2	39.2	12.5	7.0	34.6	29.2	23.3	73.0
ABT-3	33.4	31.4	14.7	50.2	29.5	9.8	10.7	43.8	24.0	16.7	69.5
MSK B2	30.2	34.7	15.8	49.8	40.7	9.6	10.3	42.4	24.4	20.5	68.7
MY 087	31.2	30.9	13.8	57.4	26.7	6.6	14.2	50.5	23.1	17.2	72.2
SED <sup>a</sup>	2.49	2.47	2.13	4.10	5.15	2.28	2.83	3.33	1.66	1.26	2.12
Sign. of effect <sup>b</sup>											
Sample	***	***	**	***	**	*	***	***	***	ns	*
Trial	***	*	*	ns	**	***	***	***	***	***	*

<sup>a</sup> SED = standard error of difference of means

<sup>b</sup> Significance of treatment effect ns = not significant; \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ .

Significant effects associated with culture were noted for all attributes except viscosity. In addition, significant effects were associated with trial, as a result - in part - of changes in the composition of the sensory panel (Table 3.3).

Notable differences in acid character - smell, flavour and aftertaste - were associated with differences in starter culture (Table 5.7). For example, fermented milk made with the AB culture was very much more acid than the corresponding product made from culture MSK 2. Products made with AB culture were also given low rating for creamy and sweet character - in contrast to those made from DV B-100, ABT-1 and MSK B2. High ratings for the attribute 'other' for yoghurt made with cultures AB and AC/BL indicated that these cultures were perceived to have flavour attributes which were not adequately described by the 10 terms in the standard vocabulary. The nature of this undefined attribute was probed by examination of the words associated with the term 'other'. The frequency of use of additional descriptors is shown in Table 5.8. Three additional terms - lemon, vinegar and chalky - were perceived to enhance the description of the flavour profile of the fermented milks. However, the use of the descriptors, lemon and chalky, was associated with all or most culture types. In contrast, the attribute vinegar was almost exclusively associated with cultures AB and AC/BL (23/27 cases). Because these cultures had significantly high ratings for 'other', it is safe to conclude that both cultures had vinegary character, albeit to different extents.

### 5.3.8.2 Multivariate analysis

Principal Component Analysis is a powerful way of summarising the main differences between objects. It is particularly useful where a large number of individual attributes differ between objects because it identifies the underlying differences in order of importance. PCA, with Factor rotation, was applied to the ratings of the 11 attributes describing the sensory character of the 24 samples of fermented milk. To compensate for systematic differences between trials, the ratings for each trial were centred (within trials) before the Factor analysis. Over 95% of variance was accounted for by 3

**Table 5.8** Association of descriptor 'other' with flavour attributes. (Trial 2)

Culture	Frequency of use of descriptor					
	Lemon	Vinegar	Chalk	Vanilla	Cream	Acrid
AB	6	19	0	0	0	3
AC/BL	10	4	4	0	2	1
DV B-100	6	0	4	2	2	1
MSK 2	2	0	6	1	1	0
ABT-1	4	0	4	0	0	0
ABT-3	4	2	5	2	0	0
MSK B2	7	1	2	4	0	1
MY 087	10	1	0	2	1	0
Total	49	27	25	11	6	6

Factors, explaining 52.0, 30.7 and 12.9% respectively. In addition, the culture effects on the scores on these 3 factors were significantly different (Table 5.9).

Sensory space maps were constructed from the Factor scores for each culture (Figures 5.8 and 5.9) together with the corresponding correlation plots to enhance interpretation of the differences between cultures. On Factors 1 and 2, cultures AB, AC/BL and MSK 2 were clearly separated from the others. MY 087 and MSK B2 were slightly separated from the grouping ABT-1, ABT-3 and DV B-100 (Fig. 5.8a). Separation on Factor 1 was mainly associated with differences in acid character, while Factor 2 resolved samples in terms of the contrast between creamy/sweet smell and 'other' (*i.e.* vinegar) flavour (Fig. 5.8b). On Factor 3, separation was on the basis of creamy/sweet flavour (Fig. 5.9b), and MY 087 was clearly separated from MSK B2 in this dimension (Fig. 5.9a). Thus, the starter cultures AB, AC/BL, MSK 2, MSK B2, MY 087 and the group comprising ABT-1, ABT-3 and DV B-100 produced fermented milks which could be distinguished in terms of sensory character. The main attributes associated with these differences were acid character, vinegar flavour and creamy/sweet character.

## 5.4 Conclusions

The production of fermented milks using single strains of bifidobacteria (*i.e.* from the National Collection and/or commercial) were unsuccessful. The products lacked the typical characteristics of a good fermented milk product, showing excessive whey separation and weak gel formation. No improvement was achieved by using the growth enhancers of bifidobacteria, for example Jerusalem artichoke flour and Raftiline.

Although the gross chemical composition of the fermented milks using commercial mixed starter cultures was similar, the profile of organic acid content varied. The fermented milks made with starter cultures AB and AC/BL showed higher rate of acetic acid production as a consequence of the highest counts of *Bifidobacterium* after the fermentation period when compared with the other products.



**Table 5.9** Principal Component Analysis with Factor Rotation (varimax)<sup>a</sup>.

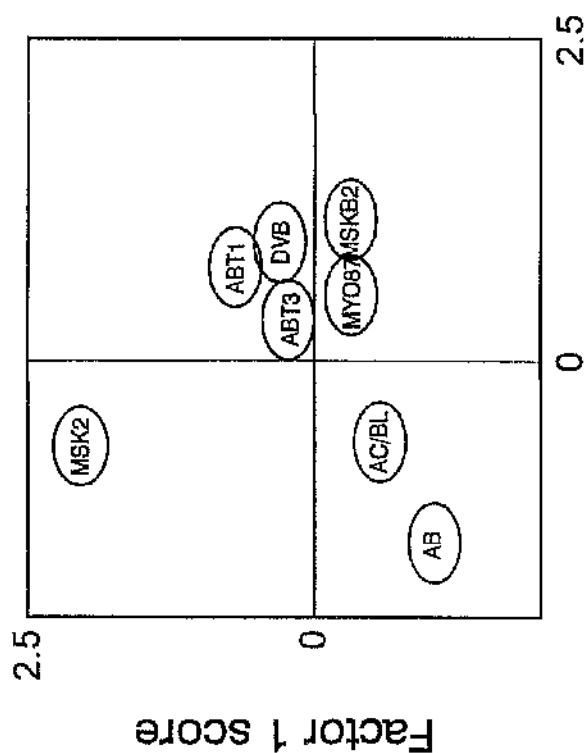
Culture	Score on Factor		
	Factor 1	Factor 2	Factor 3
AB	-1.20	-1.57	-0.37
AC/BL	-0.72	-0.77	1.39
DV B-100	0.17	0.82	0.60
MSK 2	2.02	-0.78	0.13
ABT-1	0.58	0.65	-0.13
ABT-3	0.09	0.22	-0.85
MSK B2	-0.47	1.02	0.42
MY 087	-0.47	0.42	-1.19
SED <sup>b</sup>	0.32	0.45	0.60
Variance explained, %	52.0	30.7	12.9
Sign. of culture effect <sup>c</sup>	***	***	*

<sup>a</sup> Ratings from each trial were centred before analysis. Starter culture scores, SEDs and significance of starter culture effect for first three Factors.

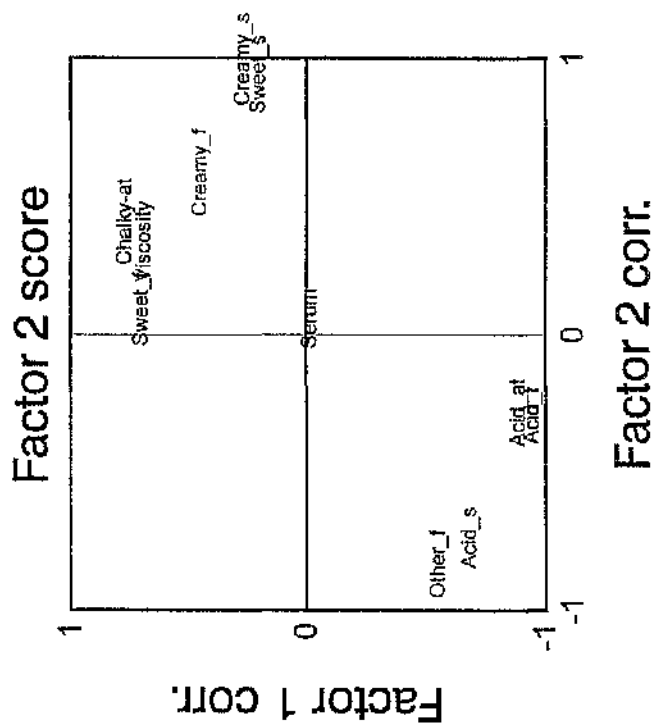
<sup>b</sup> SED = standard error of difference of means

<sup>c</sup> Significance \* =  $p < .05$ ; \*\*\* =  $p < .001$ .

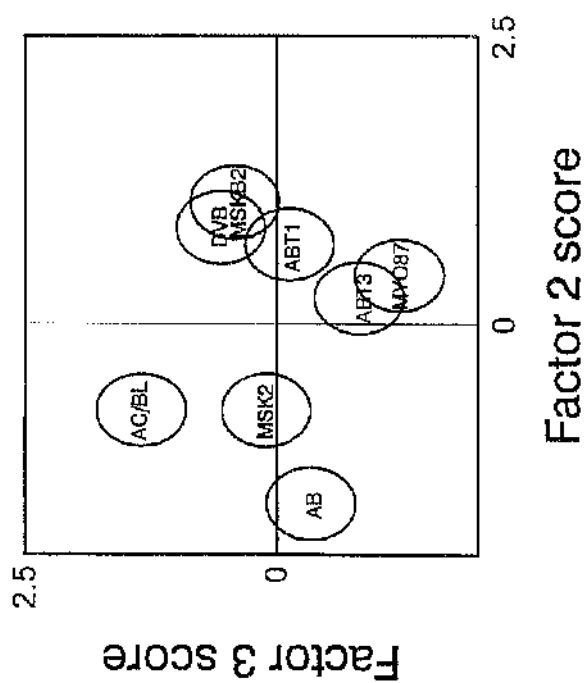
**Figure 5.8a** Sensory space for fermented milks. Factor scores on first two dimensions are shown, together with standard error ellipses. Variance explained 52.0 and 30.7%, respectively. Codes are given in text.



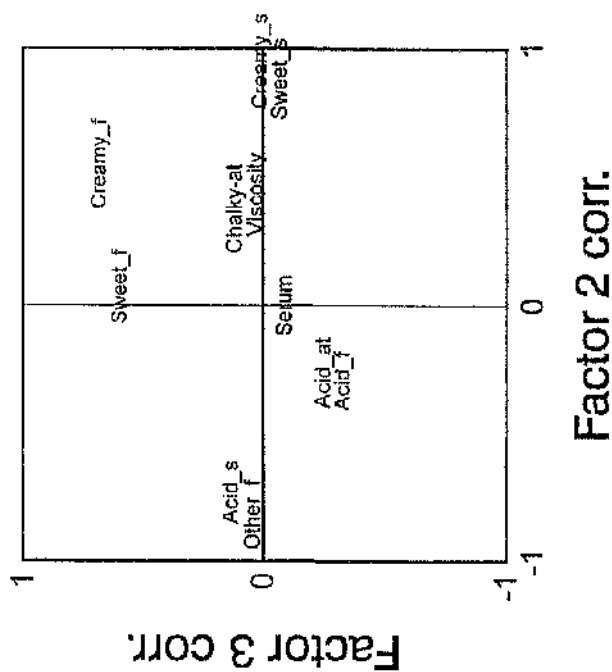
**Figure 5.8b** Interpretation of sensory space for fermented milks. Correlation of Factor scores, on first two dimensions, with original attribute ratings are shown.



**Figure 5.9a** Sensory space for fermented milks. Factor scores on third and second dimensions are shown, together with standard error ellipses. Variance explained 12.9 and 30.7%, respectively. Codes are given in text.



**Figure 5.9b** Interpretation of sensory space for fermented milks. Correlation of Factor scores, on third and second dimensions, with original attribute ratings are shown.



However, the acidification rate of the milk base varied from as low as 6h up to 22h in order to achieve the desirable pH level. The post-acidification rate of the fermented milks decreased only by around 0.2 pH units. Nevertheless, the level of acidification of all the products ensured their microbiological safety.

No processing difficulties were experienced in the production of the fermented milks as a result of: (a) the high level of  $\beta$ -Lg and  $\alpha$ -La denaturation, and (b) the appropriate level of acid development during the incubation. The microbiological quality of all the fermented milks was excellent, with the exception of two products in one of the trials made with starter cultures MSK 2 and ABT-3 which were contaminated after the heat treatment stage.

The growth of bifidobacteria was inhibited by the presence of *Str. thermophilus* in starter cultures DV B-100, MSK 2, MSK B2, ABT-1 and ABT-3. Nevertheless, despite this negative influence, and with the exception of the products made with starter cultures MSK 2 and ABT-1, the number of viable organisms after the storage period was within limits recommended by Robinson and Tamime (1993) (*i.e.*  $>10^6$  cfu  $g^{-1}$ ), for "probiotic" fermented milk. However, when bifidobacteria were grown in the presence of *Lb. acidophilus* (*i.e.* AB and AC/BL fermented milks), the viable cells counts of these micro-organisms increased suggesting an associative stimulation of growth.

The *Lb. acidophilus* viable cells counts were within acceptable levels although the fermented milk made with starter MSK B2 had a very low count after the storage period. The viable cells counts of *Lb. delbrueckii* ssp. *bulgaricus* were also within acceptable levels. However, the viable cells counts of *Str. thermophilus* were very high in all of the fermented milks even after the storage period.

Serum separation (syneresis) decreased with time in all of the fermented milks while firmness increased with time. The rate of change was lower for both, serum separation and firmness, in the initial days of storage than at the end of the storage period. However, the differences in both syneresis and firmness of the fermented milks

were statistically dependent over time ( $P < 0.001$ ). Nevertheless, the syneresis was dependent upon the type of starter culture used ( $P < 0.01$ ), but the firmness was independent of the starter culture used ( $P > 0.05$ ).

The organoleptic assessment showed significant differences associated with starter cultures for all the attributes tested, with the exception of viscosity. These results complemented those obtained from the compositional quality (*i.e.* organic acid content) and the nature of viable cells in the fermented milks. The products made with starter cultures AB and AC/BL, for example, were characterised for their high rating of the attribute "vinegar". Incidentally, these two products were high in acetic acid content and high in viable cells count of bifidobacteria. In contrast, the product made with starter culture MSK 2 was characterised as the least acid.

## **CHAPTER SIX:**

## **GENERAL DISCUSSION AND CONCLUSIONS**

## CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

The recovery of viable counts of micro-organisms in the eight different starter cultures, were according to the supplier's specifications. This suggests that the different media used for the enumeration of the starter cultures throughout the present study were satisfactory.

The morphological studies using Confocal Microscopy allowed to identify the micro-organisms at a genera level. Thus, it was confirmed that the single strain isolates belonged to bifidobacteria, lactobacilli and streptococci. The possible suggestion that *Bifidobacterium* spp. isolates from starter cultures AB, ABT-1, ABT-3 and MSK B2 may belong to *Bif. animalis*, and further physiological tests are required to confirm the identity of these organisms.

The enzymatic activity tests (API-ZYM and API 50CHL) performed on bifidobacteria isolates have been used to confirm the status of some isolates. Thus, starter cultures MSK 2, AC/BL and DV B-100 were confirmed as *Bif. longum*, *Bif. infantis* and *Bif. bifidum*, respectively. However, the ID 32A enzymatic test designed for anaerobic organisms helped to confirm the *Bifidobacterium* organisms on a genera level, but did not identify the isolates at species level. As a consequence, bifidobacteria from starter cultures AB, ABT-1, ABT-3 and MSK B2 have not been fully identified.

The enzymatic activity tests (API-ZYM and API 50CHL) performed on *Lb. acidophilus* species confirmed the identity of such organism in all of the starter cultures used. However, the isolate *Lb. delbrueckii* ssp. *bulgaricus* from starter culture MY 087 was identified as *Lb. delbrueckii* ssp. *lactis*, confirmed latter by the manufacturer.

The API-ZYM and ID32 STREP enzymatic activity tests used to characterise *Str. thermophilus* confirmed the identity of such micro-organisms.

The production of fermented milks using single strains of bifidobacteria (*i.e.* from the National Collection of Food Bacteria and/or commercial) was unsuccessful. The products lacked the characteristics typical of a good fermented milk product, and no improvement was achieved by using growth enhancers Jerusalem artichoke flour and Raftiline. The products showed excessive whey separation and weak gel formation, and most of the products did not reach the desired level of pH.

The gross chemical composition of the fermented milks using the eight commercial mixed starter cultures was similar, but the organic acid profile was different. The products made by using starter cultures containing *Lb. acidophilus* and bifidobacteria (*i.e.* AB and AC/BL) showed higher content of acetic acid, when compared with the other products containing the same organisms, in addition to *Str. thermophilus* (*i.e.* DV B-100, MSK 2, MSK B2, ABT-1 and ABT-3).

The growth of bifidobacteria was inhibited by the presence of *Str. thermophilus* in starter cultures DV B-100, MSK 2, MSK B2, ABT-1 and ABT-3. However, the number of viable organisms remained as expected after the storage period (*i.e.*  $>10^6$  cfu g<sup>-1</sup>) with the exception of the products made with starter cultures MSK 2 and ABT-1. The enumeration of bifidobacteria species and *Lb. acidophilus* using starter cultures AB and AC/BL showed a very high viable cell count (*i.e.*  $\sim 10^9$  cfu g<sup>-1</sup>) in the product (fresh and stored), suggesting a possible associative growth between them.

The viable cell counts of *Lb. acidophilus* were within acceptable levels (*i.e.*  $>10^6$  cfu g<sup>-1</sup>) after the storage period, but there was one exception where the starter culture MSK B2 showed a low levels of count after the storage period (*i.e.*  $<10^6$  cfu g<sup>-1</sup>). The count of *Lb. delbrueckii* ssp. *bulgaricus* from starter culture MY 087 showed slightly low viable cells count ( $\sim 10^6$  cfu g<sup>-1</sup>) after the storage period, this could be due to the design of this particular starter culture (*i.e.* low post-acidification). However, the viable cell counts of *Str. thermophilus* were very high for all of the products after the storage period.



The rheological properties (firmness and syneresis) followed a similar consistent pattern for all of the fermented milks. Syneresis decreased while firmness increased with time. The rate of change was slower for both firmness and serum separation, in the initial days of storage period than in the end.

The sensory evaluation provided a conclusive characterisation of the products which were positively correlated with the other tests performed on the products (*i.e.* organic acid content and enumeration of viable cells of lactic acid bacteria).

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